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Structure-function relationships of citrate synthase.

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Structure-Function Relationships

of

Citrate Synthase

submitted by M.S. Robinson
for the degree of Ph.D. of the
University of Bath
1984.

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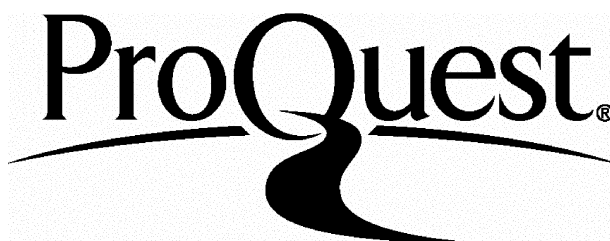
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Finally my special thanks to Carol for typing this thesis in what were far from easy circumstances. I don't know how she did it!

To the Typist

Abbreviations

Most of the abbreviations used in this thesis are those recommended in the Biochemical Society publication "Policy of the Journal and Instructions to Authors". (Biochem. J. (1984) 217, 1 - 26).

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Non-Standard Abbreviations

DEPC: Diethylpyrocarbonate

DTNB: 5,5' dithiobis-(2-nitrobenzoic acid)

OAA : Oxaloacetate

PMSF: Phenylmethanesulphonyl fluoride

TNBS: 2-nitro-5-mercaptobenzoate

'Tris buffer A' : 20mM Tris/HCl buffer, pH8.0 containing 1mM EDTA and 100mM KCl.

'Tris buffer B' : 20mM Tris/HCl buffer, pH8.0 containing 1mM EDTA, 100mM KCl and 20% (v/v) glycerol.

'Tris buffer C' : 20mM Tris/HCl buffer, pH8.0 containing 1mM EDTA.

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Summary

The purification of citrate synthase from E. coli (wild-type) is described. The subunit M_r value was determined to be approximately 47,000 by SDS-polyacrylamide gel electrophoresis. The hexameric nature of this enzyme was established using bifunctional cross-linking reagents and analysis by SDS-polyacrylamide gel electrophoresis.

The purification of citrate synthase from a strain of E. coli containing elevated levels of the enzyme is also described. This organism possesses the qlt A gene on several copies of a hybrid plasmid and the structural and regulatory properties of its citrate synthase were found to be identical to the wild-type E. coli enzyme in all aspects studied. The implications of producing elevated levels of wild-type and mutant citrate synthase are discussed.

Purification of citrate synthase from B. megaterium is reported. The enzyme was found to have a native M_r of 84,000 by a combination of analytical ultracentrifugation and gel filtration. Analysis by SDS-polyacrylamide gel electrophoresis and gel filtration under denaturing conditions revealed a subunit of M_r value 39,000 - 43,000. The enzyme was discovered to be dimeric by the use of cross-linking reagents and analysis by SDS-polyacrylamide gel electrophoresis. Modification of B. megaterium citrate synthase by chemical reagents was performed. The enzyme was insensitive to the thiol-specific reagent DTNB but inactivated by DEPC which specifically attacks histidine moieties. Spectroscopic analysis of the inactivated enzyme revealed 2 histidines modified per dimer necessary for a 100% loss

in catalytic activity. Protection against inactivation by DEPC was afforded by both substrates and ATP.

The purification of citrate synthase from a mutant strain of E. coli is described. The enzyme was found to have a native M_r value of 76,000 and a subunit M_r of 42,000 - 43,000 by gel filtration and SDS-polyacrylamide gel electrophoresis respectively.

Comparisons of the structural and functional properties of citrate synthases from various sources are discussed in the light of the sum total of these findings.

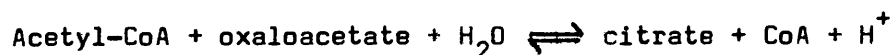
INTRODUCTION

The citric acid cycle operates in most living organisms representing a broad spectrum of diverse lifestyles. Such diversity may well be reflected in the roles that the citric acid cycle plays in order to suit the metabolic needs of a particular organism, be it provision of energy or biochemical intermediates. These roles, in turn, could possibly be reflected in finer detail in terms of the structural, catalytic and regulatory properties of the enzymic machinery of the cycle. These differences may well be particularly apparent in the enzyme catalysing the first step of the cycle - namely citrate synthase, since the initial step of a biochemical pathway often proves to be a crucial control point.

Discovery of Citrate Synthase

The citric acid cycle was first proposed by Krebs and Johnson (1937) to describe the final stages of carbohydrate oxidation by animal tissues. It is now known that this metabolic cycle is at the core of cellular metabolism and serves as the final oxidation process of all major foodstuffs in all respiring organisms (Krebs and Lowenstein, 1960).

Stern et al. (1950) first described "condensing enzyme" which brought about the combination of 'active' acetate with oxaloacetate to form citrate. 'Active' acetate was later discovered to be acetyl-CoA (Lynen and Reichert, 1951; Lynen et al., 1951) and it was thus established that "condensing enzyme", now known as citrate synthase (citrate oxaloacetate - lyase (CoA acetylating); EC4.1.3.7) catalyses the following reaction:

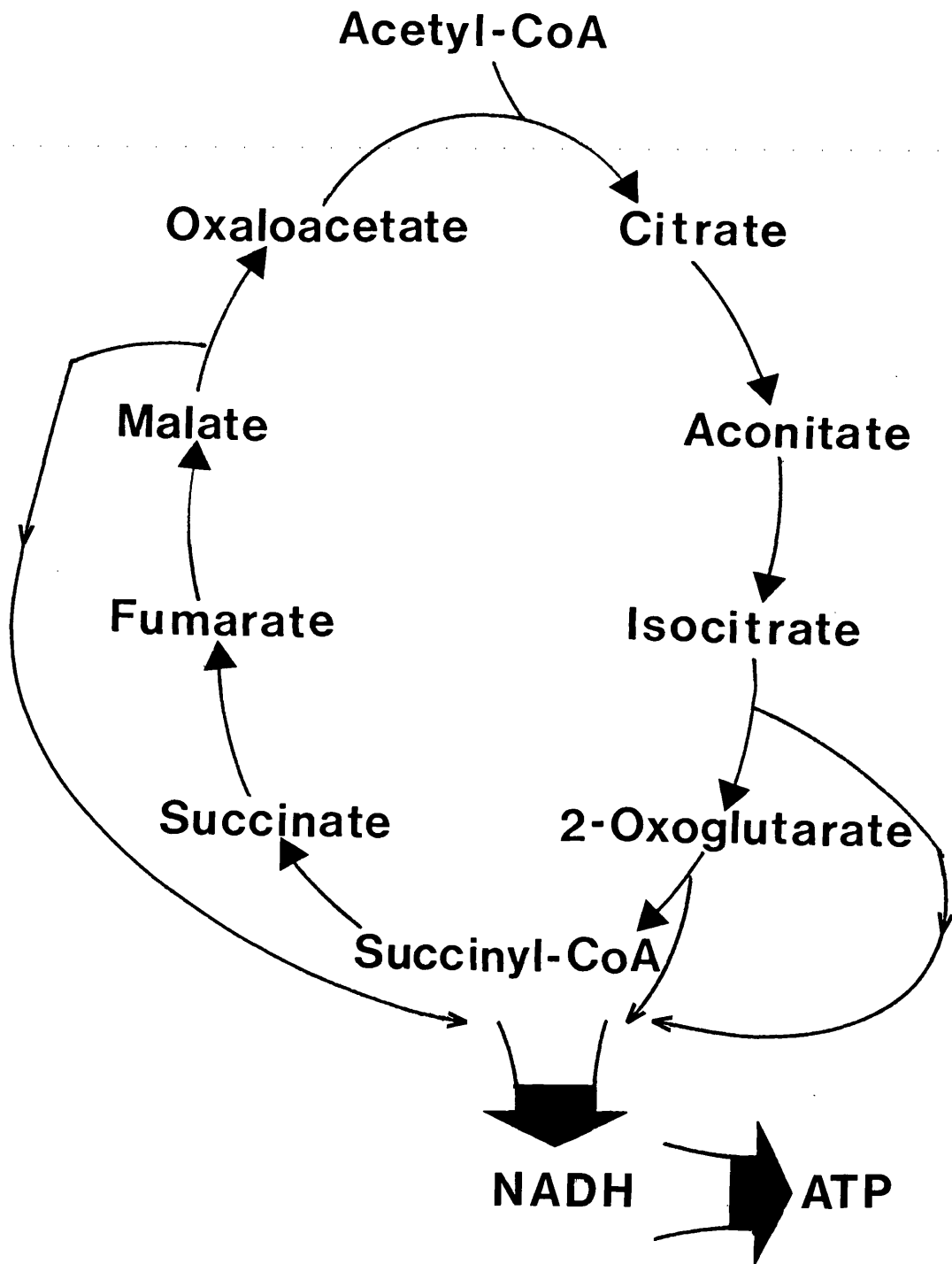


Ochoa et al. (1951) purified the enzyme from pigeon breast and moth flight muscle and showed also that citrate synthase activity could be detected in several micro-organisms, both prokaryotic and eukaryotic.

Inhibition by Nucleotides

Hathaway and Atkinson (1965) observed that citrate synthase from yeast could be inhibited in vitro by concentrations of ATP within the physiological range. This was a significant discovery since citrate synthase can be considered the 'first' enzyme of the citric acid cycle (see figure 1) and ATP one of the end products. Other eukaryotic citrate synthases are also inhibited by ATP (Bogin and Wallace, 1966; Shepherd and Garland, 1966; Jangaard et al., 1968). This inhibition was shown to be competitive with respect to acetyl-CoA. Hathaway and Atkinson (1965) also showed that ATP exerted the greatest effect while ADP and AMP were progressively weaker. It was suggested that it was the relative, rather than the absolute, effect of the three nucleotides that conferred metabolic significance. This idea has since been developed into the concept of "energy charge" (Atkinson, 1968). However, low concentrations of divalent metal ions, including Mg^{2+} , were shown to diminish the inhibition by ATP (Kosicki and Lee, 1966; Lee and Kosicki, 1967) thus casting some doubt on the physiological significance of nucleotide inhibition. In situ studies using yeast cells permeabilised by toluene (Weitzman and Hewson, 1973) and tolueinised rat liver mitochondria (Matlib et al., 1978) also showed that under these conditions ATP inhibition of citrate synthase is reduced. Inhibition of pig heart citrate synthase by Mg^{2+} was also observed

Figure 1. The Citric Acid Cycle showing the End-Product Nature
of NADH and ATP



NADH is produced by the dehydrogenation reactions of the cycle and its subsequent oxidation is used to generate the production of ATP.

(Kosicki and Lee, 1966; Lee and Kosicki, 1967) and it was suggested that these effects were achieved by the chelation of the phosphate groups of acetyl-CoA and ATP.

Citrate synthase from the Gram-negative prokaryote E. coli, however, proved relatively insensitive to ATP (Weitzman, 1966a). An alternative regulation of E. coli citrate synthase was sought and it was found that NADH acted as a powerful allosteric inhibitor (Weitzman, 1966a,b). The scheme of the citric acid cycle in figure 1 shows that NADH is produced by the later dehydrogenation reactions of the cycle and its subsequent oxidation is coupled to the production of ATP. Thus both ATP and NADH can be considered end products of the cycle.

The citrate synthase from another Gram-negative organism, Acinetobacter lwoffii also showed inhibition by NADH (Weitzman and Jones, 1968) but in this case the inhibition could be relieved by low concentrations of AMP. These findings prompted a survey of a large number of bacterial genera by Weitzman and Jones (1968). A striking pattern emerged when inhibition by NADH was investigated. The organisms could be divided into two distinct groups according to the response of their citrate synthase to NADH. The enzyme from all the Gram-negative organisms tested showed inhibition by NADH, whereas no such inhibition was observed in the citrate synthases from the Gram-positive bacteria. The Gram-negative bacterial citrate synthases fell into two subgroups based on their response to NADH. In one group, NADH inhibition was relieved by AMP and, in the other, AMP was without effect. These sub-divisions were coincident with those of the strict aerobic and facultative anaerobic organisms respectively. Weitzman and Jones (1968) suggested a rationale for

this observation based on the metabolic needs of these two classes of organism. The facultative anaerobes, as typified by E. coli, can generate energy from fermentative pathways alone without the need for the citric acid cycle, and possess glycolytic enzymes sensitive to low levels of ADP and AMP. However, the strict aerobes, typified by Acinetobacter lwoffii, are absolutely dependent on the citric acid cycle for energy production and the sensitivity of their citrate synthase to AMP may fulfill a 'low energy signal' function.

Flechtner and Hanson (1969) discovered that the citrate synthase from the Gram-positive bacterium Bacillus subtilis was inhibited by ATP, competitively with respect to acetyl-CoA. Higa and Cazzulo (1976) made similar observations using Bacillus stearothermophilus. Harford and Weitzman (1975) performed multiple-inhibitor studies and discovered that ATP competed isosterically for the acetyl-CoA binding site in all cases and that citrate synthase from Gram-negative sources was inhibited allosterically by NADH.

Several other inhibitors of citrate synthase have been discovered. Wieland and Weiss (1963) observed that palmitoyl-CoA, the end product of fatty acid synthesis, inhibited mammalian citrate synthase. However the interaction was shown to be a non-specific detergent type (Srere, 1965) since 16 molecules of palmitoyl-CoA bound to each enzyme molecule.

Inhibition by 2-Oxoglutarate

Citrate synthase from E. coli was found to be inhibited by 2-oxoglutarate (Wright et al., 1967) and the allosteric nature of the inhibition was indicated by the desensitization brought about by KCl or high pH. This prompted Weitzman and Dunmore (1969) to investigate the inhibition by 2-oxoglutarate of a number of citrate synthases

from various organisms. Once again, a distinct pattern emerged. Of the enzymes tested, only those from facultatively anaerobic Gram-negative bacteria were sensitive to 2-oxoglutarate. No inhibition was displayed by the enzymes from aerobic Gram-negative bacteria, Gram-positive bacteria or eukaryotic organisms.

Observations by Amarasingham and Davis (1965) had suggested that when E. coli was grown anaerobically the citric acid cycle was modified to the branched non-cyclic pathway shown in figure 2, with an apparent absence of the enzyme 2-oxoglutarate dehydrogenase. Under these circumstances citrate synthase fulfills a biosynthetic role in the production of 2-oxo glutarate which, in turn, is used for the biosynthesis of amino acids. The end-product nature of 2-oxoglutarate is apparent in this scheme, and its inhibitory action on citrate synthase is characteristic of feedback inhibition by an end-product on the initial step of its production.

The Molecular Sizes and Subunit Composition of Citrate Synthase

The variation in regulatory behaviour of citrate synthase from various sources and the allosteric nature of the NADH inhibition of the enzyme from Gram-negative organisms prompted Weitzman and Dunmore (1969) to investigate the molecular sizes of a variety of citrate synthases. These studies were based on the rationale that allosteric behaviour is mediated by a specific arrangement of subunits in the quaternary structure of an enzyme. Electron micrographic observations (Rowe and Weitzman, 1969) had indicated that citrate synthase from the Gram-negative aerobe Acinetobacter lwoffii was polymeric and the enzyme exhibited conformational changes in the presence of NADH and AMP.

Using zonal gel filtration Weitzman and Dunmore (1969) observed

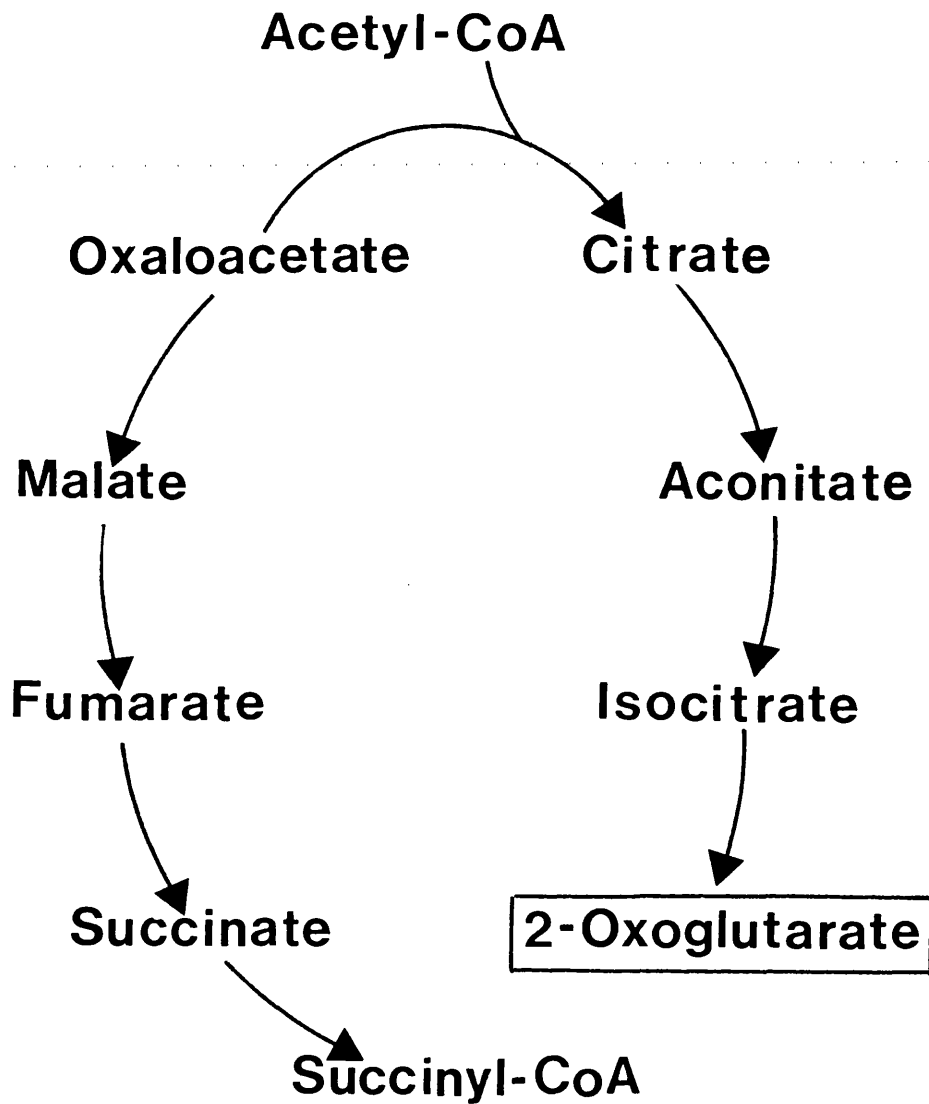


Figure 2. The Modified Citric Acid Cycle for Anaerobic Growth and End-Product Inhibition of Citrate Synthase by 2-Oxoglutarate

that the citrate synthases from various sources fell into two distinct categories: one with M_r values of approximately 250,000 termed 'large'; and the other with M_r values of approximately 100,000 termed 'small'. Not only were there two distinct groups based on size but all the 'large' enzymes studied were sensitive to NADH while all the small enzymes were observed to be insensitive to NADH but inhibited by ATP.

The subunit composition of both 'small' and 'large' citrate synthases has been investigated. The 'small' enzyme from eukaryotic sources has been purified and shown to be a dimer (Wu and Yang, 1970; Singh et al., 1970; Moriyama and Srere, 1971; Wiegand et al., 1979), with apparently identical subunits having M_r values in the range 46,000 - 58,000 (Shepherd and Garland, 1969; Singh et al., 1970; Wu and Yang, 1970; Moriyama and Srere, 1971). However the 'small' enzyme from Gram-positive bacteria has not been studied in any detail and there are no reliable reports of either subunit size or composition.

The 'large' citrate synthase from E. coli has undergone considerable scrutiny. Wright and Sanwal (1971) reported that the enzyme was a mixture of tetramers and octamers with a subunit M_r value of approximately 62,000. Danson and Weitzman (1973) suggested that the enzyme was tetrameric based on a value of M_r 230,000 for the native protein and a subunit M_r value of 55,000. Observations by Tong and Duckworth (1975), using a cross-linking reagent, however, suggested that the enzyme was hexameric. Evidence from peptide mapping (Wright and Sanwal, 1971) and N-terminal analysis (Tong and Duckworth, 1975) indicated that the subunits were identical. However the subunit number of the 'large' citrate synthase is yet to be resolved.

The Identification of Amino Acid Residues in Catalysis and Regulation

Crystal structure analysis of citrate synthase from pig heart (Wiegand et al., 1979) showed that the individual polypeptide chains were folded predominantly as helices in two domains. The citrate binding site was located but the corresponding site for CoA was elusive and lay in a disordered part of the enzyme molecule. However, useful information regarding the roles played by particular amino acid residues in the catalytic and regulatory properties of the enzyme has been obtained using chemical reagents which specifically modify certain amino acid side chains.

Using 5,5'-dithiobis (2-nitrobenzoate), DTNB, a reagent that specifically modifies thiol groups, Danson and Weitzman (1973, 1977) suggested that cysteine residues played an important role in both the regulation and catalytic activity of the 'large' citrate synthase from E. coli. DTNB was capable of modifying 8 thiol groups per enzyme molecule; two thiols were modified rapidly with some 26% loss of catalytic activity and 6 more slowly resulting in a further 60% reduction in activity. Of these 6 thiols, 4 were involved in NADH inhibition and loss of NADH sensitivity was obtained only when at least 3 of these thiols were modified. No loss of sensitivity to 2-oxoglutarate was observed.

The eukaryotic citrate synthases are, on the whole, insensitive to DTNB, although some plant enzymes undergo modification and in-activation (Srere et al. 1971 ; Weitzman and Danson, 1976).

Histidine side chains have been implicated in both the catalytic and regulatory functions of citrate synthase. Using photo-oxidation studies of E. coli citrate synthase, several amino acid residues were modified resulting in a loss of catalytic activity and desensitization

to both NADH and 2-oxoglutarate (Danson and Weitzman, 1973). The pH profile of the loss of catalytic activity indicated that histidine residues were undergoing modification and Danson and Weitzman (1973) suggested that the photo-oxidizable residue at the 2-oxoglutarate effector site was also probably histidine. Danson and Weitzman (1973) also observed loss of 80% of catalytic activity following modification of 2 - 3 histidines per subunit ($M_r=57,000$) using diethylpyrocarbonate (DEPC).

The citrate synthase from pig heart also underwent modification and inactivation by DEPC and it was discovered that the ethoxy-formylation of 2 - 3 histidines per enzyme molecule caused a 50% loss in catalytic activity (Mählen, 1975). Mählen (1975) also observed that some protection against DEPC-inactivation was provided by oxaloacetate.

Some Unusual Citrate Synthases

Few exceptions to the patterns observed by Weitzman and Jones (1968) and Weitzman and Dunmore (1969) have been discovered. Swissa and Benziman (1976) reported that the citrate synthase from Acetobacter xylinum is insensitive to NADH although the organism is Gram-negative. However the enzyme is of the 'large' type in keeping with the Gram staining behaviour of Acetobacter.

Some examples of citrate synthase exist which, at first sight, do not appear to fall into the categories discussed earlier, but nevertheless possess structural and functional properties to suit the metabolic needs of the organisms in which they occur. As illustrated previously Gram-positive bacterial citrate synthases are 'small'. There exists another group of bacteria which are not Gram-positive but also possess 'small' citrate synthases. Examples of these organisms are the halobacteria (Cazzulo, 1973; Weitzman and Danson 1976;

Weitzman, 1978). They represent a category of organisms known as Archaeobacteria (Woese, 1981). In keeping with their 'small' size, the enzymes are insensitive to NADH. The Archaeobacteria may well resemble examples of the earliest forms of life on earth and they are characteristically found in Nature in extreme conditions of high temperature and high salinity. In the case of the halophiles, Cazzulo (1973) suggested that as inhibition of citrate synthase by NADH is overcome by high salt concentrations in all cases, the evolutionary adaptation to halophilic habitats has resulted in the loss of this mode of regulation which has been rendered ineffective by these conditions.

The citrate synthase extracted from the gills of abyssal fish Antimora rostrata, shows structural properties suited to the environment of the fish (Hochachka, 1975). When observed under normal laboratory conditions this eukaryotic citrate synthase existed uncharacteristically as a 'large' enzyme with a value of M_r of 270,000. However when subjected to the conditions of the habitat of the fish, namely 200 - 250 atmospheres pressure, the enzyme appeared to depolymerize into a more active dimeric enzyme of M_r 100,000 in keeping with its eukaryotic origin (Hochachka, 1975).

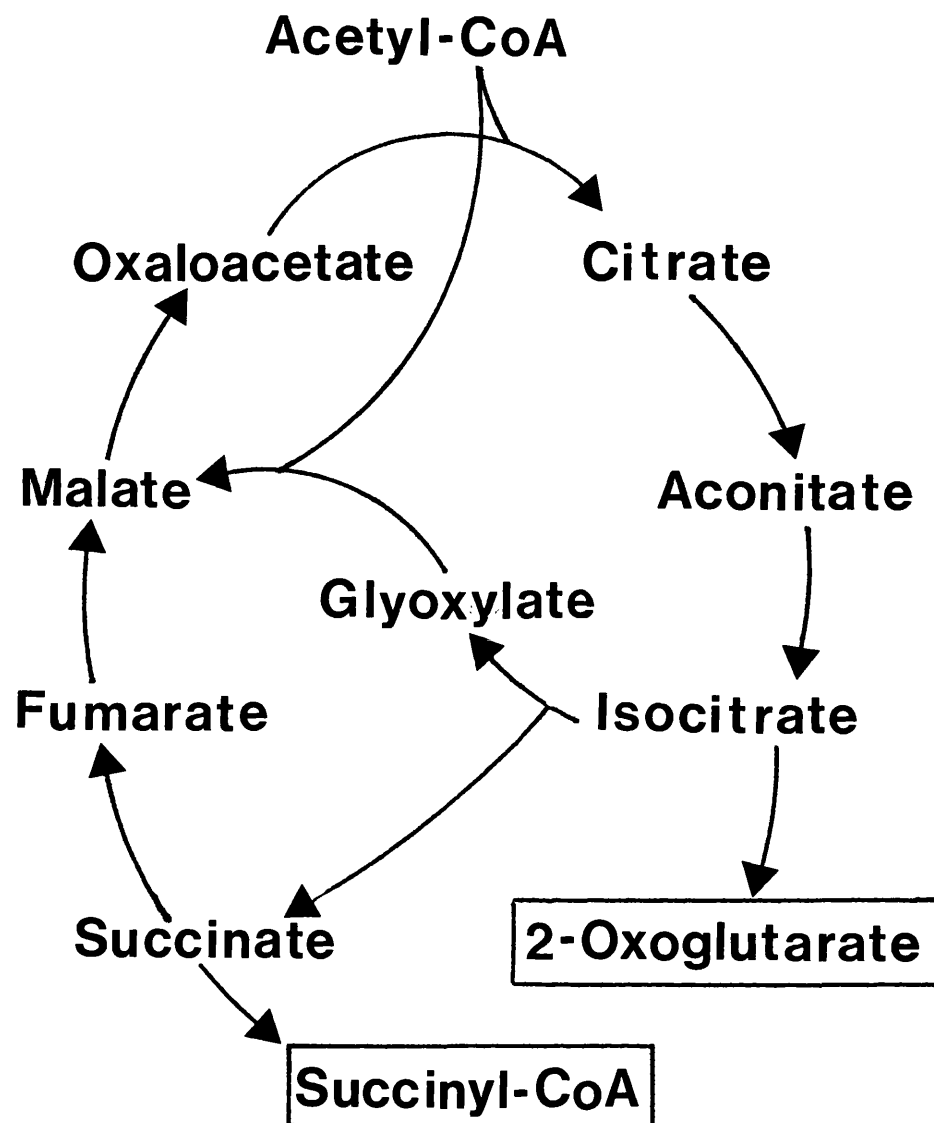
A further example of a citrate synthase whose properties reflect its metabolic role is to be found in the cyanobacteria. These Gram-negative obligate phototrophs lack the enzyme 2-oxoglutarate dehydrogenase (Pearce and Carr, 1967; Smith et al., 1967; Pearce et al., 1967). This absence of a complete energy-yielding citric acid cycle prompted studies on the regulation of the cyanobacterial citrate synthase. Inhibition of the enzyme was achieved by 2-oxoglutarate (Taylor, 1973; Lucas and Weitzman, 1975) but NADH proved

ineffective as an inhibitor (Taylor, 1973; Lucas and Weitzman, 1975). This insensitivity to NADH suggested a 'small' type of citrate synthase. However examination of the molecular size of this enzyme revealed a 'large' citrate synthase (Lucas and Weitzman, 1975). The enzyme was also inhibited powerfully and specifically by succinyl-CoA (Lucas and Weitzman, 1977). This observation followed the discovery that succinyl-CoA is formed in cyanobacteria not by the split pathway shown in figure 2 but via the operation of the glyoxylate cycle (Pearce et al., 1969) as presented schematically in figure 3. Thus citrate synthase functions as the first enzyme in the pathway leading to the formation of both 2-oxoglutarate and succinyl-CoA.

Mutant Citrate Synthases

So far the structure - function relationships of citrate synthases from naturally occurring/wild-type diverse organisms have been discussed. However a novel, complementary approach has been used in which mutant organisms are generated possessing citrate synthases with altered structural and regulatory properties (Harford and Weitzman, 1978; Weitzman et al., 1978; Danson et al. 1979b). This has been achieved with E. coli (Harford and Weitzman, 1978) and Acinetobacter lwoffii (Weitzman et al., 1978). In studies with E. coli, a citrate synthase-deficient strain (auxotrophic for glutamate) was allowed to revert spontaneously and the revertant strains which had regained citrate synthase activity were studied. Apart from apparently wild-type revertants, a 'large' NADH-insensitive enzyme (resembling Acetobacter citrate synthase) was discovered and perhaps, even more interestingly, a 'small', NADH-insensitive, ATP-sensitive citrate synthase which resembled the enzyme from eukaryotic sources and

Figure 3 The Glyoxylate Cycle and Modified Citric Acid Cycle
Operating in Cyanobacteria



Succinyl-CoA and 2-oxoglutarate act as end-product inhibitors of citrate synthase from cyanobacteria.

Gram-positive bacteria. It also displayed hyperbolic responses to its substrates, unlike the wild-type enzyme which exhibits sigmoidal substrate dependences. Harford and Weitzman (1978) concluded that a minor genetic alteration had occurred to bring about a drastic change in the nature of the enzyme. Subsequent genetic mapping studies of the 'small' mutant by Danson et al. (1979b) revealed that the gene coding for the 'small' enzyme was a modified form of the wild-type gene coding for the 'large' enzyme. Further information on the relationships and differences between 'large' and 'small' citrate synthases will be forthcoming only when purification of the 'small' enzyme from both a Gram-positive bacterial source and a mutant Gram-negative bacterium has been achieved, and only then will the polymeric nature of these enzymes be established. The uncertainty concerning the subunit size and number of the E. coli wild-type 'large' citrate synthase also remains to be resolved.

Aims of the Present Work

This thesis concerns itself with comparing and contrasting 'large' and 'small' citrate synthases in finer detail, particularly from bacterial sources. To these ends the purification is described of the citrate synthase from both wild-type E. coli and the mutant strain carrying the 'small' enzyme. Investigation of their respective subunit compositions has been carried out using a combination of cross-linking reagents and determination of the subunit M_r values. The purification and analysis of a 'small' citrate synthase from a Gram-positive organism is also described. The organism selected for this work was Bacillus megaterium, a spore forming Gram-positive aerobe, and the purification of its citrate synthase is reported

for the first time. Finer detail concerning functional and essential amino acid side chains is provided by chemical modification with specific reagents. The properties of both Gram-negative, Gram-positive and eukaryotic citrate synthases are discussed in the light of these observations.

Complementary studies of the citrate synthase gene from various sources are continuing in Professor Weitzman's and Dr Danson's group at Bath, and elsewhere, and together with the information gained by protein chemistry may provide evidence for the particular function of certain amino acid residues in terms of catalytic, regulatory and structural characteristics of citrate synthase from diverse sources.

Organisms

Escherichia coli, strain CA244 was glycerol-grown and obtained as a frozen paste from the Microbiological Research Establishment, Porton Down, Wilts., U.K.

Escherichia coli, strain JA200/pLC26-17 (F^+ thr leu lac Y₁ trp E5 rec A₁) was kindly provided by Professor J.R. Guest (University of Sheffield). The organism contained several copies of a synthetic ColE1 - E. coli hybrid plasmid, designated pLC26-17 incorporating the E. coli citrate synthase (glt A) gene (Guest, 1981).

Escherichia coli, strain K114R (Hfr pps met thy str^S) was from the culture collection of Professor P.D.J. Weitzman (University of Bath).

Bacillus megaterium, strain D101 was from the culture collection of the Department of Microbiology, University of Leicester, Leicester, U.K.

Materials

Chemicals used were analytical grade or the finest grade commercially available. Tryptone and yeast extract were from Difco Laboratories, Detroit, Michigan, U.S.A.; Oxoid nutrient broth and MacConkey agar were from Oxoid Limited, London, U.K.; coenzyme A, oxaloacetate, NADPH, pig heart pyruvate kinase, rabbit muscle fructose biphosphate aldolase, rabbit muscle glyceraldehyde 3- phosphate dehydrogenase, pig heart citrate synthase, pig heart lactate dehydrogenase, pig heart fumarase, pig heart malate dehydrogenase, bovine liver catalase, yeast triose phosphate isomerase and rabbit muscle phosphorylase a were from Boehringer, Mannheim, Germany; 5,5'-dithiobis-(2-nitrobenzoic acid), glutaraldehyde (aqueous 25% solution), phenylmethanesulphonyl fluoride, bovine serum albumin, horse heart cytochrome c, whale skeletal-muscle myoglobin, E. coli β -galactosidase, 2-mercaptoethanol, 2-oxoglutarate, thymine, L-methionine, ATP, ADP, AMP, NADP⁺, NAD⁺, NADH and Coomassie Brilliant Blue were from Sigma Chemical Co., Poole, Dorset, U.K., dimethylsuberimide and mercaptoethanesulphonic acid were from Pierce Chemical Co., Rockford, IL, U.S.A., diethylpyrocarbonate was from BDH Chemicals Ltd., Poole, Dorset, U.K.; protamine sulphate (salmon roe) was from Koch-Light, Colnbrook, Bucks., U.K.; bromophenol blue was from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.; lysozyme (egg-white) was from Miles Laboratories, Elkhart, IN, U.S.A.; DEAE-Sephacel, Sephadex G-200, Sephacryl S-200 (superfine grade), Sephacryl S-300 (superfine grade), CNBr-activated Sepharose 4B, Mono Q and Blue Dextran 2000 were from Pharmacia;

DEAE-cellulose was from Whatman Chemical Separation Ltd., U.K.; Matrex Gels Red A, Blue B and Orange A were from Amicon Corp., Lexington, MA, U.S.A.; guanidinium chloride was from Fluka, Buchs, Switzerland; iodo [2-¹⁴C] acetic acid was from Amersham International, Amersham, Bucks., U.K.

Acetyl-CoA was prepared using CoA and acetic anhydride by the method of Stadtman (1957) and E. coli pyruvate dehydrogenase complex was purified by the method of Danson et al., (1979a).

METHODS

Growth and Harvesting of Organisms

E. coli JA200 and B. megaterium were grown aerobically at 37°C at pH 7.2 in L Broth of composition: tryptone (10g/l), yeast extract (5g/l) and NaCl (5g/l) (Lennox, 1955).

E. coli K114R was grown aerobically at 37°C in Oxoid nutrient broth supplemented with methionine (20mg/l) and thymine (20mg/l).

Cells were harvested either by means of the Alpha-Laval Separator LAB 102B-05 in the clarification mode or by centrifugation at 20,000g (r_{av} 11.9 cm) for 15 min. The cells were then re-suspended in the appropriate buffer, recentrifuged and stored frozen at -20°C.

Assay of Citrate Synthase

Method 1

Citrate synthase was assayed spectrophotometrically at 412 nm at 25°C by the method of Srere et al. (1963). Unless otherwise stated the assay mixture contained (final concentrations) 20mM Tris/HCl buffer, pH 8.0, 1mM EDTA, 0.15mM acetyl-CoA, 0.2mM oxaloacetate and 0.1mM DTNB. The reaction was initiated by adding the enzyme to bring the total volume to 1.0ml. The CoA produced reacts with DTNB to form the yellow coloured thio-nitrobenzoate anion (TNBS⁻). The molar extinction coefficient of TNBS⁻ was taken as 13,600 l.mol.⁻¹ cm⁻¹ at pH 8.0 (Ellman, 1959).

Method 2

A second spectrophotometric assay of citrate synthase activity

involves measurement of the decrease in extinction at 232 nm accompanying the cleavage of the S-acyl bond in acetyl-CoA (Srere and Kosicki, 1961). Unless otherwise stated the assay mixture contained (final concentrations) 50mM sodium acetate buffer, pH 6.5, 1mM EDTA, 0.15mM acetyl CoA and 0.2mM oxaloacetate. The reaction was initiated by the addition of enzyme to bring the final volume to 1.0ml.

Determination of Protein Concentration

The concentration of protein in impure preparations was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard and in purified preparations from the values of absorbance at 260 nm and 280 nm. (Layne, 1957).

Specific activity is expressed as μmol of CoA produced/min/mg of protein.

Gram Stain

The Gram stain was performed as described by Gillies and Dodds (1973). A loopful of the bacteria under investigation was spread on a microscope slide. The slide was dried in air and then passed slowly 3 times through a bunsen flame in order to 'fix' the film of material. The slide was flooded with Methyl Violet, left for 5 min and then rinsed with Gram's iodine. After a further 2 min the slide was drained and the cells decolourised by exposure to acetone for 5 s before washing in water. Neutral Red was applied as a counter-stain and after 30 s the slide was rinsed with water and blotted dry.

Ehrlich's Test

Ehrlich's test for indole was performed as described by Collins and Lyne (1976). A culture of bacteria was grown in tryptone broth at 44°C for 48h. A solution of Ehrlich's reagent was prepared by dissolving 0.4g of 4-dimethylaminobenzaldehyde in a mixture of 8ml of 11M HCl and 38ml of ethanol. A few drops of xylene were added to the broth culture which was shaken gently. The culture was then treated with a few drops of Ehrlich's reagent and reshaken. The production of a rose-pink colour indicates the presence of indole.

Preparation of Cell-Free Extracts

Frozen cells were thawed and suspended in a volume of the appropriate buffer to give a ratio of 1g cells per 2ml buffer. This and all subsequent steps were carried out at 4°C. For extracts of volume 10ml or more, the suspension was passed twice through a pre-cooled French press at a pressure of 62 MPa (9000 lbf/in²). The cell debris was removed by centrifugation for 30 min at 20,000g (r_{av} 11.9cm).

For extracts less than 10ml in volume it was more convenient to disrupt the cells by sonication. This was performed on ice using the Ultrasonics Rapidis Sonicator with the 5mm probe at a power of 50W. Three 20s bursts were applied, interspersed with 15s intervals to allow the extract to re-cool. Cell debris was removed by centrifugation as before.

Treatment with Protamine Sulphate

Nucleic acid was removed from cell-free extracts by the dropwise addition of a 2% (w/v) aqueous solution (1mg protamine sulphate per 20mg protein) and the suspension was stirred gently for 15 min. The precipitated nucleic acid was removed by centrifugation for 30 min at 20,000g (r_{av} 11.9cm).

DEAE-Cellulose Chromatography

DEAE-cellulose was prepared for use as described in the "Whatman Advanced Ion-exchange Celluloses Laboratory Manual". A column of bed dimensions 31cmx3cm was poured and equilibrated with the appropriate buffer. The enzyme extract was applied to the column which was then washed with buffer until no more protein was eluted. Citrate synthase was eluted using a linear gradient of KCl in buffer.

DEAE-Sephacel Chromatography

A column of bed dimensions 35cmx3cm was poured and equilibrated with the appropriate buffer. Enzyme application, washing and elution were carried out as described above for DEAE-cellulose.

Fast Protein Liquid Chromatography (Pharmacia)

Ion-exchange chromatography was performed on the Pharmacia FPLC system using the Mono Q column which is an anion exchanger. Partially purified enzyme extracts were applied and citrate synthase was eluted using gradients of Cl^- or CH_3COO^- as counterions in various buffers.

Ion-Exchange Chromatography using Matrix Gel Red A

Matrix Gel Red A was prepared for use according to the manufacturers recommendations in "Operating Instructions-Matrex Gels, Amicon".

Columns of various bed dimensions were used and equilibrated with the appropriate buffer. After application of the enzyme extract the column was washed with buffer and citrate synthase eluted with a linear gradient of NaCl in buffer.

Affinity Chromatography using Matrex Gels

Matrex Gels Red A, Blue B and Orange A were prepared for use according to the manufacturers recommendations in "Operating Instructions-Matrex Gels, Amicon". Columns of bed size 8cmx0.5cm were poured and equilibrated with the appropriate buffer. The enzyme extract was applied and the column washed with buffer. Citrate synthase was eluted in buffer containing 0.1mM oxaloacetate and 0.1mM CoA.

Affinity Chromatography using Sepharose-ATP

Sepharose-ATP was prepared by the method of Lamed et al. (1973). CNBr-activated Sepharose 4B (1g) was swelled on a glass sinter with 200ml of 1mM HCl and then added to 3.5ml of cold, saturated adipic acid dihydrazide (90g/l), in 0.1M sodium carbonate buffer, pH9.5. The mixture was left overnight, stirring at 4°C and then washed thoroughly, first with water, and then 0.2M NaCl. ATP (11mg/ml) was neutralized with NaOH and 1ml was mixed with an equal volume of 20mM sodium metaperiodate and left to oxidize for 1h in the dark at 4°C. The Sepharose hydrazide was resuspended in 2ml of 0.1M sodium acetate, pH5.0, and added to 10µmol (1ml) of the oxidized ATP. The volume was made up to 5 ml with 0.1M sodium acetate, pH5.0 and the mixture was stirred for 3h at 4°C. After adding 15ml of 2M NaCl, stirring was continued for a further 30 min. This slurry was then used to pour a column (bed size 7cmx0.5cm) which was washed thoroughly with water before use.

The column was equilibrated with the appropriate buffer before the enzyme extract was applied. After washing the column with buffer, enzyme elution was performed using buffer containing 0.1mM oxaloacetate and 0.1mM CoA.

Gel Filtration

Gel filtration was carried out on various matrices either as a means of increasing enzyme purity or for the determination of M_r values. All matrices were prepared for use as described in the manufacturers' instructions.

(a) Non-Denaturing Conditions

Gel filtration on Sephadex G-200 was carried out using the appropriate buffer at a flow rate of 10ml/h.

Sephacryl S-200 and Sephacryl S-300 (both superfine grades) were used with appropriate buffers at flow rates of 10-20 ml/h.

(b) Denaturing Conditions

Gel filtration of dissociated enzymes on Sephacryl S-300 (superfine grade) in 6M guanidinium chloride was performed by the method of Kresze *et al.* (1980), as described by Danson and Porteous (1981). A 6M solution of guanidinium chloride was stirred with 1% (w/v) activated charcoal for 2h at room temperature, followed by suction filtration through Whatman No.1 filter paper. Final clarification was achieved by filtration through a Sartorius filter, pore size 0.45 μ m. Standard proteins and citrate synthase (50 μ g) were dissolved in 0.5ml of 0.1M Tris/HCl buffer, pH 8.5 containing 6M guanidinium chloride and 2mM EDTA. A freshly prepared aqueous solution of 0.1M dithiothreitol (10 μ l) was added and the solution flushed with nitrogen, sealed and incubated for 30min at 30°C. A 20 μ l portion of 54mM iodo(2-¹⁴C)acetic acid (4.7 μ Ci/ μ mol) was added, the vessel re-flushed with nitrogen, sealed and incubated at 30°C in the dark for 1-2h. A drop of 2-mercaptoethanol was added to react with excess iodoacetic acid before application to the column.

A column (bed size 76cmx1.6cm) of Sephacryl S-300 was equilibrated with 6M guanidinium chloride, pH 5.0, at room temperature. Blue Dextran and 2-nitro 5-mercaptobenzoate (TNBS) were used as markers

for the void volume and total volume respectively. The flow rate was maintained at 5ml/h and 0.5ml fractions were collected. Samples (50 μ l) of each fraction were added to 0.2ml of water in 3ml of toluene /triton 2:1 (v/v) containing 2,5 diphenyloxazole (5g/l) as scintillant. The ^{14}C content of each fraction was counted on a Packard Tri-Carb Scintillation Spectrometer for 10 min.

Analytical Ultracentrifugation

Sedimentation-velocity analyses were performed in a Beckman L5-50B analytical ultracentrifuge. Migration of the boundary was followed at a wavelength of 280nm with a U.V. scanner and the observed sedimentation coefficients in the various buffers used were corrected to the density and viscosity of water at 20°C ($s_{20,w}$ values) as described by Bowen (1970a).

High-speed sedimentation-equilibrium studies were carried out according to Yphantis (1964). The partial specific volume (\bar{v}) was calculated from the amino acid composition as described by Bowen (1970b).

Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis at pH8.9 was performed as described by Davis (1964) using 7.5% (w/v) polyacrylamide gels prepared in 9cmx0.5cm glass tubes. A sample solution of 10 μ g of purified protein in 50 μ l of 20mM Tris/HCl buffer, pH8.0 containing 1mM EDTA and 10% (w/v) sucrose was coloured with 0.001% (w/v) Bromophenol Blue and then applied to the gels. The sample was allowed to enter the gel at 0.5 mA per gel and then run at 2mA per gel until the marker dye had travelled approximately 8cm. The protein was visualised by

staining with 1% (w/v) Coomassie Brilliant Blue in 12.5% (w/v) tri-chloroacetic acid according to Chrambach et al (1967).

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Electrophoresis in the presence of sodium dodecyl sulphate (SDS) was performed by two methods.

(a) Method 1-Continuous

The first method described is based on that used by Shapiro et al. (1967). The gels were prepared in 9cmx0.5cm glass tubes using 0.1M sodium phosphate buffer, pH6.7, containing 7.5% (w/v) acrylamide (unless otherwise stated), 0.2% (w/v) bisacrylamide (unless otherwise stated), 0.1% (w/v) SDS, 0.05% (v/v) N,N,N',N'-tetramethyl-1,2-diaminoethane and 0.1% (w/v) ammonium persulphate. The gels were then overlaid with 0.1% (w/v) SDS solution and allowed to set. The buffer used in both upper and lower compartments of the electrophoresis apparatus was 0.1M sodium phosphate, pH6.7, containing 0.1% (w/v) SDS.

Purified protein samples in 20mM Tris/HCl, pH8.0, containing 0.1mM EDTA were dried down under vacuum in acid-washed glass tubes. The samples were resuspended in 0.1M sodium phosphate buffer, pH6.7, containing 1% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol and 0.001% (w/v) Bromophenol Blue as a marker dye. The final protein concentration was approximately 0.5µg/ml. The solution was heated to 100°C in a water bath for 90s. Approximately 30µl of the protein solution were then applied to each gel. The samples were then allowed to enter the gels at a current of 2mA per gel after which electrophoresis was continued at 6mA per gel until the marker dye had travelled approximately 8cm.

The gels were fixed overnight at 37°C in methanol:acetic acid:water in the ratios 5:1:5. Staining was carried out at 37°C for 45

min using the fixing solution containing 0.1% (w/v) Coomassie Brilliant Blue. Gels were destained at 37°C in methanol:acetic acid:water in the ratio 2:3:35.

SDS-polyacrylamide gel electrophoresis was carried out using this continuous method at pH6.7 unless otherwise stated.

(b) Method 2-Discontinuous

The second method used is that described by Laemmli (1970). Electrophoresis in the presence of SDS was performed at pH8.9 in a discontinuous buffer system using Tris/HCl buffer, pH8.9, in the gel, and Tris/glycine buffer, pH8.3, in the upper and lower reservoirs of the electrophoresis apparatus.

Purified protein was dried down as before and the sample resuspended in 0.1M Tris/HCl, pH8.9, containing 1% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, and 0.001% (w/v) Bromophenol Blue. This solution was then heated in a water bath as before, and a 30µl sample, representing 5-10µg of protein, was applied to each gel.

The gels were prepared in 9cm x 0.5cm glass tubes using 0.375M Tris/HCl buffer, pH8.9, containing 7.5% (w/v) acrylamide, 0.2% (w/v) bisacrylamide, 0.1% (w/v) SDS, 0.05% (v/v) N,N,N',N'-tetramethyl-1,2-diaminoethane and 0.1% ammonium persulphate. The gels were overlaid with 0.1% (w/v) SDS and allowed to set. The reservoir buffer, pH8.3, was 5mM Tris, 38mM glycine and 0.1% (w/v) SDS. The sample was allowed to enter the gel at 0.5mA per gel after which electrophoresis was continued at 2mA per gel until the marker dye had run approximately 8cm. Fixing, staining and destaining were carried out as described in Method 1.

Amino Acid Analysis

Protein samples were reduced and S-carboxymethylated with iodoacetic acid as described by Gibbons and Perham (1970). Solutions of citrate synthase in the appropriate buffer were dried down under vacuum in acid-washed glass tubes, 50µg of protein per tube. The protein was dissolved in 1ml of 0.1M Tris/HCl buffer, pH8.5, containing 5M guanidinium chloride. A 20µl portion of freshly prepared 0.1M dithiothreitol solution was added, the tube flushed with nitrogen, resealed and incubated in the dark at 30°C for 30 min. A drop of 2-mercaptoethanol was added and the sample dialysed overnight against 50mM Tris/HCl buffer, pH8.5, containing 2M urea. Finally the sample was dialysed overnight against water and dried down under vacuum.

Hydrolysis was carried out in sealed, evacuated tubes using 200µl of 6M HCl (Aristar grade) for 24, 48 and 72h at 105°C. The samples were then dried down and taken up in 200µl of 25mM HCl for analysis using a Rank Hilger Chromaspek J180 amino acid analyser. Values for tryptophan were obtained by hydrolysis for 24h in 200µl of 3M mercaptoethanesulphonic acid at 105°C. After hydrolysis the sample was treated with 200µl of 1M NaOH, mixed, and then 200µl of this neutralized sample were taken for analysis. Values for threonine and serine were corrected for loss by extrapolation to zero time. For isoleucine and valine the 72h values were used.

Protein Modification

(a) Cross-Linking using Bifunctional Reagents

Cross-linking with dimethylsuberimide was performed by the method of Davis and Stark (1970). Pure enzyme, of concentration no more than 40µg/ml, was incubated for 3h at room temperature in 2ml

of 0.2M triethanolamine/HCl buffer, pH8.5, containing 24mg of dimethylsuberimide.

Cross-linking with glutaraldehyde was performed by the method of Nucci et al. (1978). Pure enzyme (10µg/ml) was incubated for 3h at room temperature in 0.05M sodium phosphate buffer, pH7.5, containing 0.25% (v/v) glutaraldehyde, 1mM 2-mercaptoethanol and 20% (v/v) glycerol.

Samples of cross-linked protein were dialysed against 20mM Tris/HCl buffer, pH8.0, containing 1mM EDTA, before being analysed by SDS-polyacrylamide gel electrophoresis.

(b) DTNB

Pure citrate synthase was incubated in the dark at a concentration of 0.1mg/ml with various concentrations of DTNB in the appropriate buffer. The change in absorbance was monitored at 412nm over a period of time. Samples were withdrawn at intervals and assayed for citrate synthase activity.

(c) Diethylpyrocarbonate (DEPC)

The concentration of diethylpyrocarbonate (DEPC) in commercial preparations was determined from the increase in absorbance at 240nm with excess (0.3mM) imidazole, pH8.0, on the basis of a molar absorption coefficient for the product, N-ethoxyformylimidazole of $3,200 \text{ l. mol}^{-1} \text{ cm}^{-1}$ according to Ovádi et al (1967).

Pure citrate synthase was used at a concentration of 50µg/ml in 1ml of 50mM sodium acetate buffer, pH6.5, containing 20% (v/v) glycerol. DEPC was added at various concentrations as a freshly prepared aqueous solution and incubations were performed at 4°C. Samples were removed periodically for assay of citrate synthase activity. Ethoxyformyl histidine was detected by scanning a difference spectrum of modified enzyme versus native, untreated enzyme from 280nm to 220nm

using a Pye Unicam SP8-100 dual beam spectrophotometer.

Treatment of the modified enzyme with hydroxylamine was performed as follows. The ethoxyformylated protein was dialysed extensively against 20mM phosphate buffer, pH7.0, containing 20% (v/v) glycerol and then incubated in the same buffer with 0.25M hydroxylamine at room temperature for 30 min. The enzyme was then dialysed extensively against 20mM Tris/HCl buffer, pH8.0, containing 1mM EDTA and 20% (v/v) glycerol before being assayed for citrate synthase activity.

CHAPTER 1

The Citrate Synthase from *E. coli* CA244

(Wild-Type)

The Citrate Synthase from *E. coli* CA244 (Wild-Type)

Introduction

The regulatory properties of citrate synthase from *E. coli* have been well characterised (Weitzman and Danson, 1976; Weitzman, 1980). However there is some doubt as to the structural composition of the enzyme from this Gram-negative bacterial source. Evidence based on the amino acid composition together with peptide mapping studies suggests that the subunits may be identical (Wright and Sanwal, 1971) and indeed the enzyme has been shown to behave as a single molecular species under SDS-polyacrylamide gel electrophoresis (Wright and Sanwal, 1971; Danson and Weitzman, 1973; Tong and Duckworth, 1975). There is general agreement about the value of M_r for the native enzyme and estimates are in the range 230,000 - 270,000 (Wright and Sanwal, 1971; Danson and Weitzman, 1973; Tong and Duckworth, 1975). Previous studies on the M_r values of the subunits have suggested that the enzyme is tetrameric with subunit M_r values of 60,000 - 65,000 (Wright and Sanwal, 1971) and 55,000 - 57,000 (Danson and Weitzman, 1973). These estimates were based on results obtained from SDS-polyacrylamide gel electrophoresis. However, using both sedimentation-equilibrium centrifugation in guanidinium chloride and SDS-polyacrylamide gel electrophoresis, Tong and Duckworth (1975) suggested that the subunits have an M_r value of 43,400 - 47,000. After cross-linking the native enzyme with dimethylsuberimidate, analysis on SDS-polyacrylamide gel electrophoresis revealed 6 bands whose apparent M_r values were multiples of 47,000 (Tong and Duckworth, 1975).

The objectives of the present work were thus two-fold: First,

to establish a reliable method of estimating the value of the subunit M_r and second, to determine the number of subunits comprising the native enzyme.

The first objective may be approached by testing the reliability of determinations of M_r values using a method described by Banker and Cotman (1972). This analysis allows the measurement of two molecular parameters that together, determine the migration rate of protein molecules undergoing SDS-polyacrylamide gel electrophoresis. These parameters are the retardation coefficient (K_R) and the free electrophoretic mobility (M_o). The retardation coefficient is dependent solely on molecular size and is related to the sieving properties of the gel, in this case polyacrylamide. The free electrophoretic mobility is a function of the charge and size of a molecule. If protein molecules bind SDS uniformly (1.4 g of SDS per g of protein, Reynolds and Tanford, 1970) then a direct relationship should exist between the size of a molecule and its net charge. Hence M_o and K_R should be related linearly for such proteins. Both quantities may be determined for any given protein undergoing electrophoresis in the presence of SDS, by measuring migration rates (M) in a series of gels of varying polyacrylamide concentration (T) from the equation first proposed by Ferguson (1964):

$$\log M = \log M_o - K_R T$$

Ferguson plots of $\log M$ versus T will permit the determination of M_o and K_R . Secondary plots of the values determined for M_o versus the values determined for K_R can be constructed.

The second objective, the determination of the subunit number, may be achieved by exposing the native enzyme to bifunctional reagents which have the ability to cross-link the subunits together covalently.

The cross-linked enzyme can then be analysed using SDS-polyacrylamide gel electrophoresis, the number of bands on each gel being the number of subunits. These then were the strategies employed to investigate the structural properties of wild-type E. coli citrate synthase.

Results

Assay of E.coli Citrate Synthase

The most sensitive and convenient assay was that performed spectrophotometrically at 412nm as described in Methods. The assay buffer used was 20mM Tris/HCl, pH 8.0 containing 1mM EDTA and 100mM KCl. This buffer is hereafter referred to as 'Tris Buffer A',

Enzyme Purification

E. coli citrate synthase was purified by adapting a method used by Danson and Weitzman (1973).

Following treatment with protamine sulphate the enzyme was applied to a column (3cm x 30cm) of DEAE-cellulose. The column was washed with 500ml of 'Tris buffer A' before eluting citrate synthase with a linear gradient of 0.1M - 0.5M KCl in 500ml of 'Tris buffer A'. The effluent was collected in fractions (7ml) at a flow rate of 15ml/h and the enzyme was recovered in those fractions containing approximately 0.25M KCl. Fractions containing citrate synthase at a specific activity greater than 1 unit/mg were pooled and diluted 1:3 with 20mM Tris/HCl, pH8.0, 1mM EDTA to bring the final KCl concentration to approximately 50mM.

A column (25cm x 1.3cm) of Matrex Gel Red A was prepared and equilibrated with 20mM Tris/HCl, pH8.0, containing 1mM EDTA

and 50mM KCl. The pooled, selected, diluted fractions from the previous step were applied to this column which was then washed with 300ml of the equilibration buffer, and citrate synthase was eluted with this buffer containing 0.1mM oxaloacetate and 0.1mM CoA. The eluent was collected in fractions (2ml) at a flow rate of 10ml/h and citrate synthase was recovered after approximately 15ml. Those fractions containing the enzyme at a specific activity greater than 20 units/mg were pooled giving a total volume of 4ml.

This solution was then applied to a column (3cm x 35cm) of Sephadex G-200 previously equilibrated with 'Tris buffer A'. Citrate synthase was eluted in the same buffer in fractions (2.5ml) at a flow rate of 10ml/h. Fractions containing the enzyme at a specific activity greater than 45 units/mg were analysed using SDS-polyacrylamide gel electrophoresis and found to run as a single component as shown in the gel scan in figure 4.

A summary of the purification procedure is presented in table 1. This 5-step purification procedure represents a 500-fold increase in specific activity and gave a 14% overall yield. The enzyme was stable for at least 3 months in 'Tris buffer A' when stored at a concentration of 50µg/ml at 4°C.

Determination of the Subunit M_r

The enzyme was subjected to continuous SDS-polyacrylamide gel electrophoresis in phosphate buffer, pH6.7. The following standard proteins were also run: E.coli pyruvate decarboxylase, E. coli lipoate acetyltransferase, E. coli dihydrolipoamide dehydrogenase, rabbit muscle fructose biphosphate aldolase and rabbit muscle glyceraldehyde 3-phosphate dehydrogenase. The

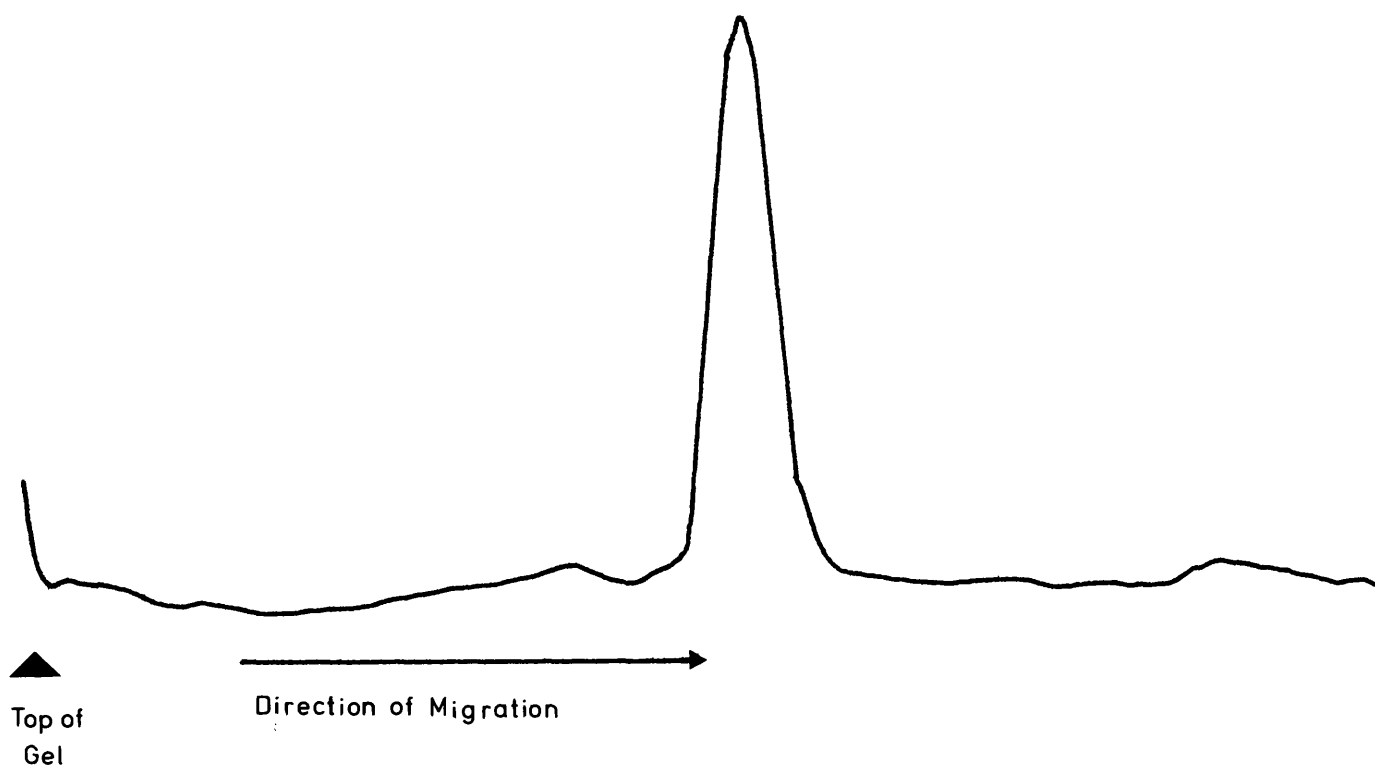


Figure 4. Densitometer Trace of Purified Citrate Synthase from

E. coli CA244 after SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis at pH6.7 and staining were performed as described in Methods.

Table 1. Purification of Citrate Synthase from E. coli CA244

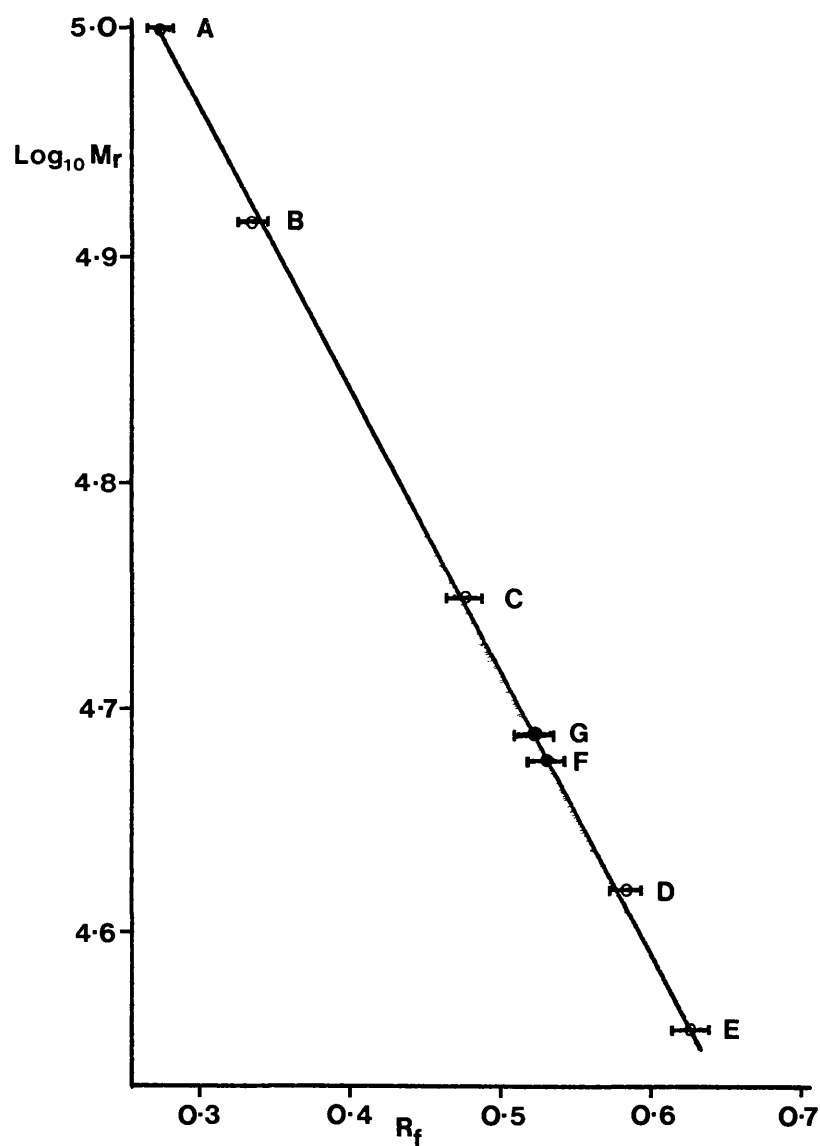
Step	Volume (ml)	Total citrate synthase (units)	Total protein (mg)	Specific activity (units/mg)	Yield %
(1) French press extract	50	285	2700	0.1	-
(2) Supernatant from protamine SO ₄ treatment	48	283	1884	0.2	99
(3) Pooled,selected fractions from DEAE-cellulose ion exchange	49	221	223	1.0	78
(4) Pooled,selected fractions from Matrex Gel Red A -elution with OAA and CoA.	3	126	6.4	19.7	44
(5) Pooled,selected fractions from Sephadex G-200	18	38.9	0.8	49.9	14

results plotted as $\log M_r$ versus R_f (mobility relative to marker dye) are shown in figure 5. E. coli citrate synthase ran as a single molecular species with a determined M_r of 46,800 ($\pm 1,000$).

In order to test the validity of this result a series of Ferguson plots was constructed as follows. E. coli citrate synthase was subjected to electrophoresis together with other proteins as standards on gels of differing polyacrylamide concentration (T) in the presence of SDS and phosphate buffer, pH6.7. The electrophoretic mobility (M) was measured relative to that of whale skeletal-muscle myoglobin as described by Vogel (1977). Ferguson plots ($\log M$ versus T) are shown in figure 6. The proteins used as standards were: E. coli β -galactosidase, E. coli pyruvate decarboxylase, E. coli lipoate acetyltransferase, E. coli dihydrolipoamide dehydrogenase, rabbit muscle fructose bisphosphate aldolase and pig heart lactate dehydrogenase. For each protein, values for the free electrophoretic mobility, M_0 (determined from the intercept on the ordinate) and the retardation coefficient, K_R (determined from the gradient) were calculated. The values of K_R were plotted against the respective values of M_0 and the results are shown in figure 7. A direct linear relationship was found to exist between M_0 and K_R indicating that the proteins migrate according to their respective molecular weights under these conditions. The co-ordinates representing K_R and M_0 for E. coli citrate synthase also lie on this line indicating that the migration distance of the enzyme is a true reflection of its size at pH6.7. It is therefore concluded that the subunit M_r of E. coli citrate synthase determined by this method is a reliable estimate.

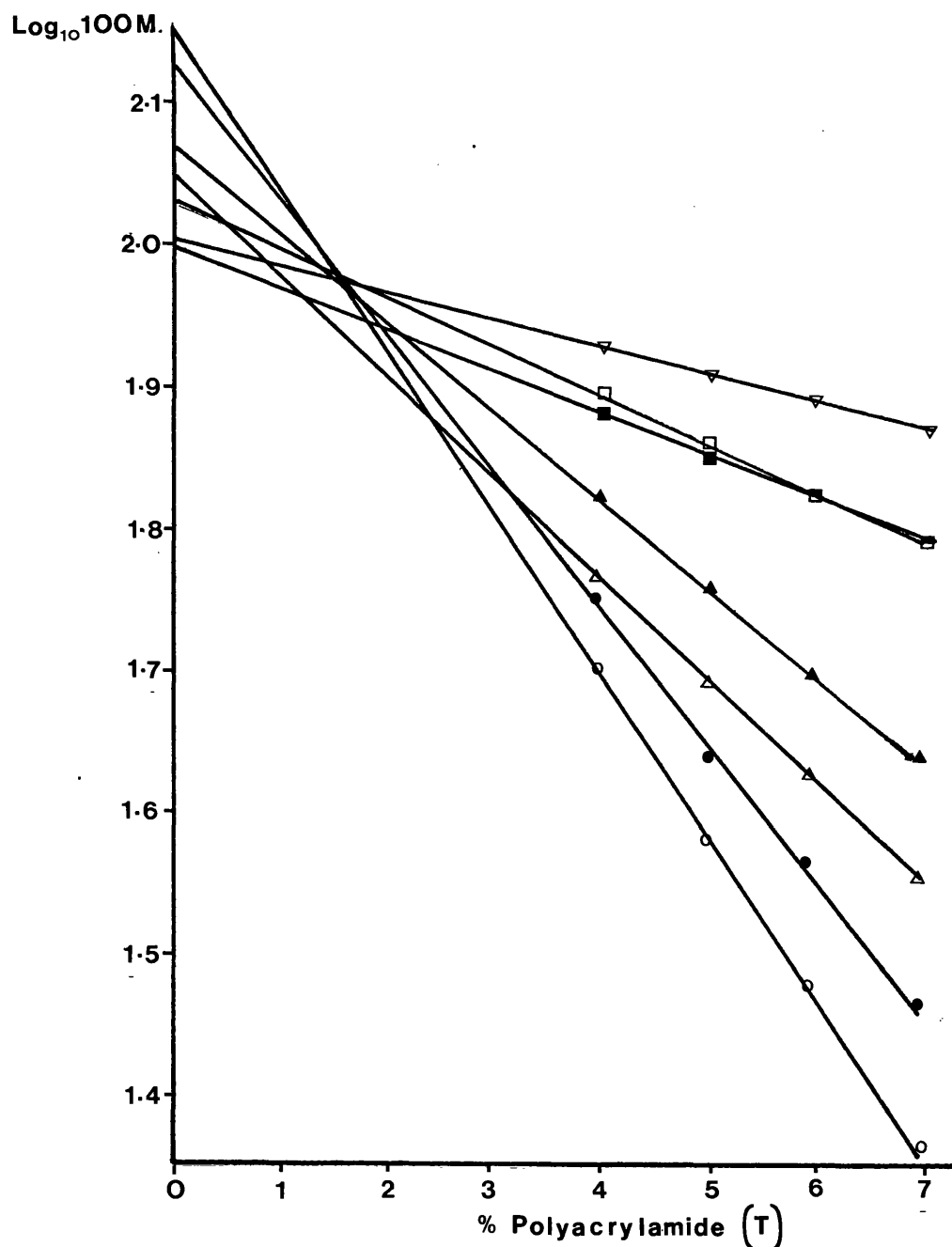
E. coli citrate synthase was subjected to SDS-polyacrylamide

**Figure 5. SDS-Polyacrylamide Gel Electrophoresis at pH6.7 -
Relationship Between $\log_{10} M_r$ and Relative Mobility (R_f)**



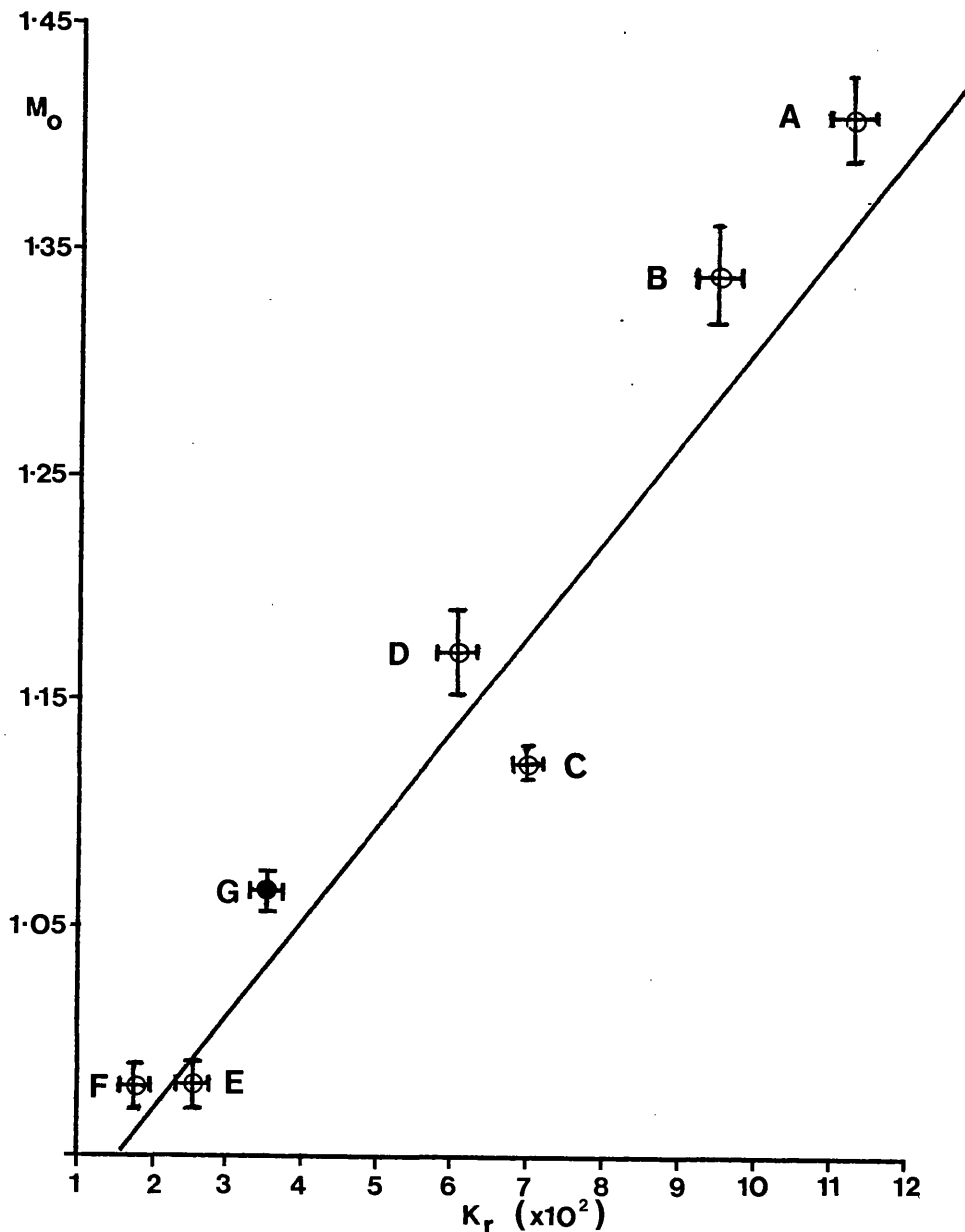
SDS-polyacrylamide gel electrophoresis was performed at pH6.7 using the continuous system described in Method 1. Proteins used as standards were: (A), E. coli pyruvate decarboxylase (M_r 100,000); (B), E. coli lipote acetyltransferase (M_r 83,000); (C) E. coli lipoteamide dehydrogenase (56,500); (D) rabbit muscle fructose bisphosphate aldolase (M_r 42,000); (E) rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (M_r 36,000). (F) represents citrate synthase from E. coli CA244 and (G) represents citrate synthase from pig heart, The bars represent the S.E.M. of the mobilities, relative to marker dye, of each protein in three separate determinations.

Figure 6. Ferguson Plots for Various Proteins Subjected to SDS-Polyacrylamide Gel Electrophoresis at pH6.7 - Relationship between $\log_{10} 100 M$ and % Polyacrylamide (T)



SDS-polyacrylamide gel electrophoresis was performed on various proteins on gels of varying % of polyacrylamide as described in the text. Proteins used were: (O), *E. coli* β-galactosidase (M_r 120,000); (●), *E. coli* pyruvate decarboxylase (M_r 100,000); (Δ), *E. coli* lipoate acetyltransferase (M_r 83,000); (▲), *E. coli* lipoamide dehydrogenase (M_r 56,500); (□), *E. coli* CA244 citrate synthase; (■), rabbit muscle fructose biphosphate aldolase (M_r 42,000); and (▽), pig heart lactate dehydrogenase (M_r 36,000).

Figure 7. SDS-Polyacrylamide Gel Electrophoresis at pH6.7 -
Relationship between Free Electrophoretic Mobility
(M_0) and Retardation Coefficient (K_r)



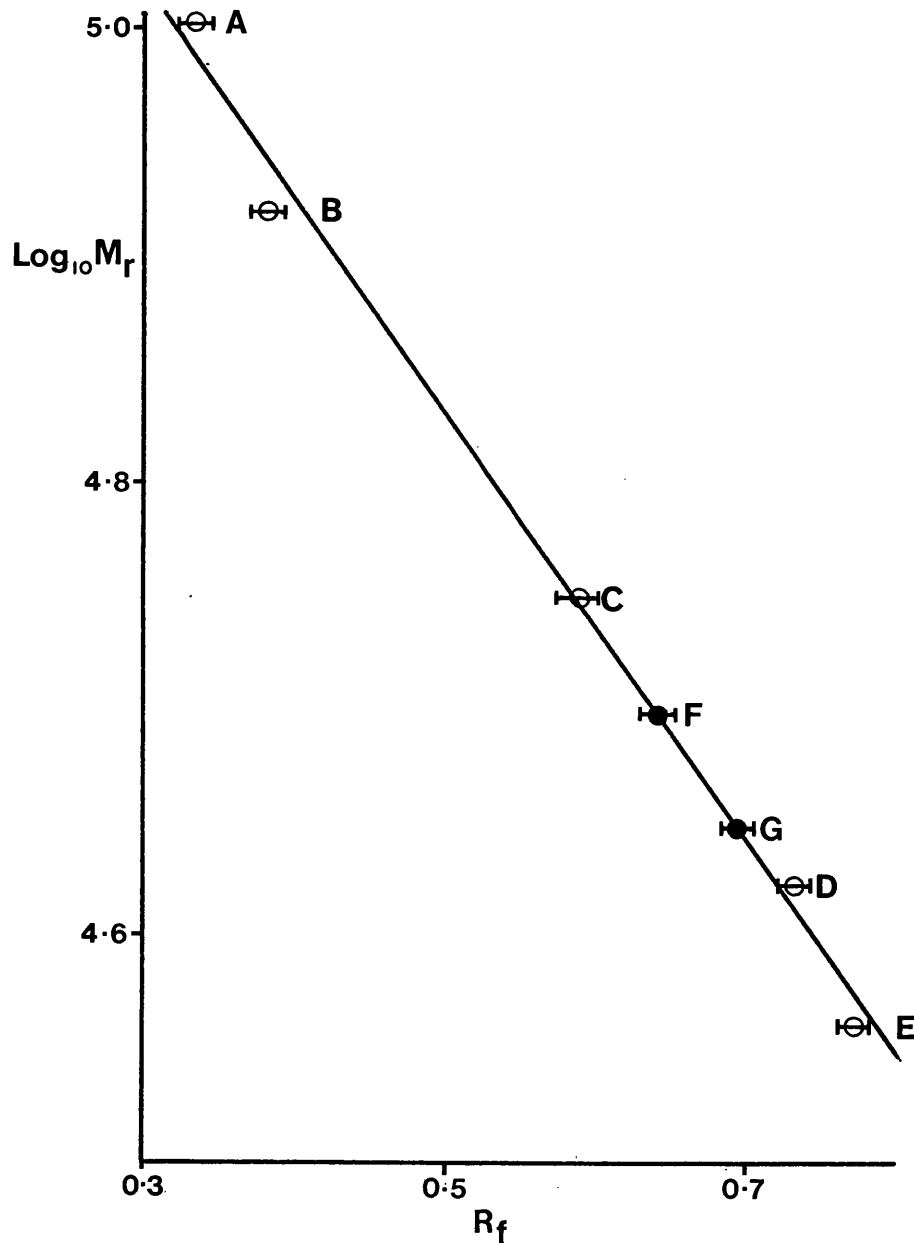
The values of M_0 and K_r and their respective S.E.M. values (represented by bars) were determined for each protein from the Ferguson plots in figure 6 as described in the text. The proteins are represented as follows: (A), E. coli β -galactosidase; (B), E. coli pyruvate decarboxylase; (C), E. coli lipoate acetyltransferase; (D), E. coli lipoamide dehydrogenase; (E), rabbit muscle fructose biphosphate aldolase; and (F) pig heart lactate dehydrogenase. (G) represents E. coli CA244 citrate synthase.

gel electrophoresis at pH8.9 using the discontinuous Tris-glycine system as described in Methods. Proteins used as standards were: E. coli β -galactosidase, E. coli pyruvate decarboxylase, E. coli lipoate acetyltransferase, E. coli dihydrolipoamide dehydrogenase, rabbit muscle fructose bisphosphate aldolase and pig heart lactate dehydrogenase. Mobilities were determined relative to that of the marker dye. Pig heart citrate synthase was also run for comparison. The results are presented in figure 8, plotted as $\log M_r$ versus Rf (relative retardation coefficient). The values determined for the subunit M_r of citrate synthase from pig heart and E. coli were 44,300 ($\pm 1,000$) and 49,400 ($\pm 1,000$) respectively. Apparently the subunit of E. coli citrate synthase behaved in this system as though it were larger than that from the pig heart enzyme. When the corresponding experiment was carried out using the continuous phosphate buffer, pH6.7, system (figure 5) the Rf of the E. coli enzyme was greater than that of the enzyme from pig heart, that is the relative mobilities were inverted when compared to the Tris-glycine system, figure 8. The Rf values determined at pH 6.7 using phosphate buffer corresponded to subunit M_r values of 49,000 ($\pm 1,000$) and 46,800 ($\pm 1,000$) for the pig heart and E. coli citrate synthase respectively.

The unreliability of the Tris-glycine system was confirmed when Ferguson plots were constructed with data from SDS-polyacrylamide gel electrophoresis at pH8.9. Plots of M_o versus K_R showed no direct linear relationship (C.G. Mitchell, personal communication).

As a result of these studies all subsequent M_r determinations using SDS-polyacrylamide gel electrophoresis were carried out using the continuous system with phosphate buffer, pH6.7.

Figure 8. SDS-Polyacrylamide Gel Electrophoresis at pH8.9 -
Relationship between $\log_{10} M_r$ and Relative Mobility (R_f)



SDS-polyacrylamide gel electrophoresis was performed at pH8.9 using the discontinuous system described in Method 2. Proteins used as standards were: (A), E. coli pyruvate decarboxylase (M_r 100,000); (B), E. coli lipoate acetyltransferase (M_r 83,000); (C), E. coli lipoamide dehydrogenase (M_r 56,500); (D) rabbit muscle fructose biphosphate aldolase (M_r 42,000); and (E), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (M_r 36,000). (F) and (G) represent citrate synthase from E. coli CA244 and pig heart respectively. The bars represent S.E.M. of the mobilities, relative to marker dye, of each protein in three separate determinations.

Determination of the Number of Subunits

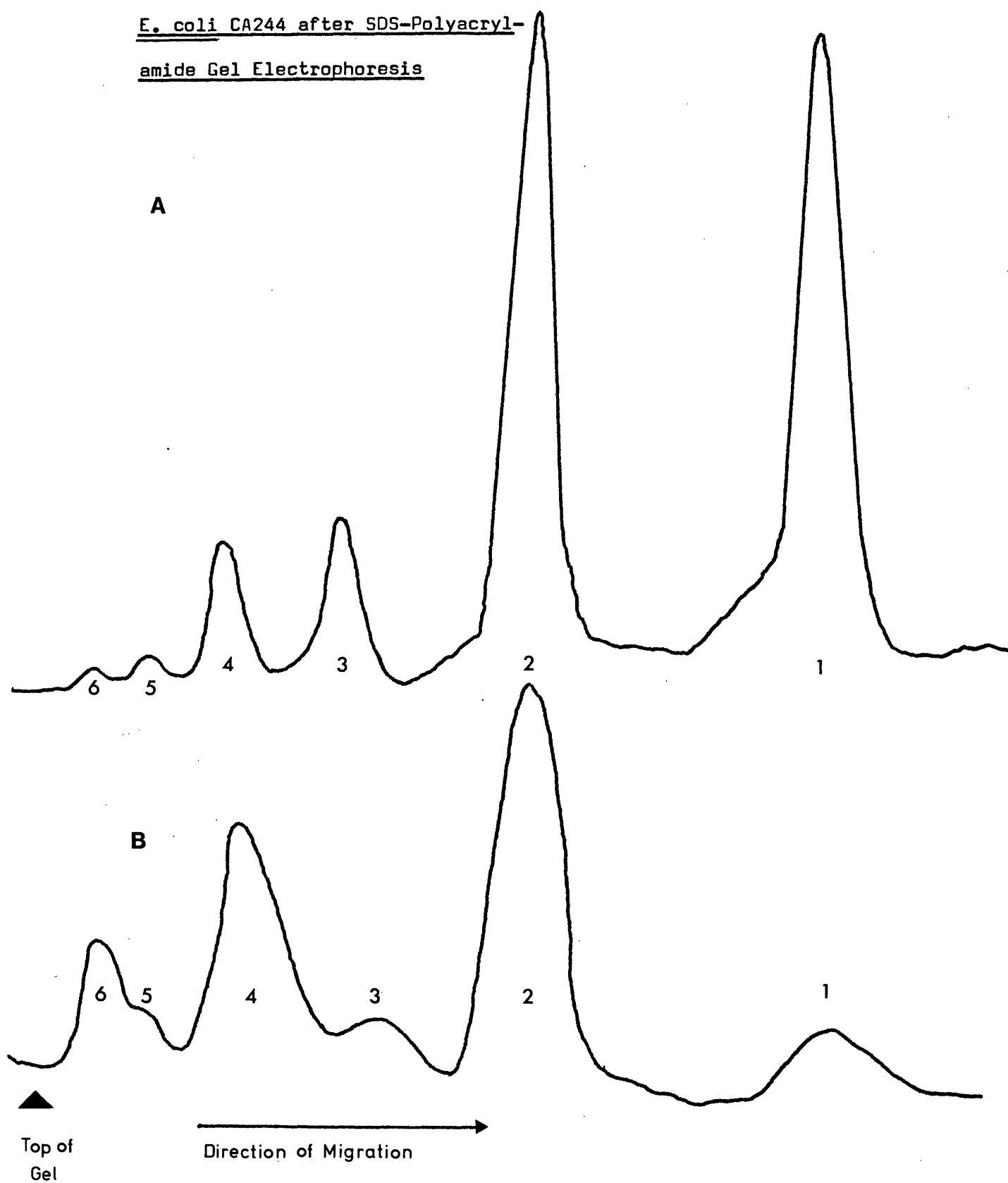
E. coli citrate synthase was cross-linked using either dimethylsuberimide or glutaraldehyde in the presence of 100mM KCl as described in Methods. Cross-linked enzyme was subjected to electrophoresis in the presence of SDS on 5% (w/v) polyacrylamide gels and the gels were scanned using a Pye Unicam SP 1800 spectrophotometer. The results are shown in figure 9. With either reagent, six protein bands were detected having relative approximate M_r values of 6:5:4:3:2:1. No molecular species greater than hexamer was detected. When dimethylsuberimide was used, relatively little of the higher M_r species was detected even after 6h incubation. The cross-linking pattern obtained using glutaraldehyde was somewhat different. Relatively low proportions of the monomer, trimer and pentamer were produced and the dimeric, tetrameric and hexameric species predominated.

Discussion

The evidence from SDS-polyacrylamide gel electrophoresis and cross-linking suggests that E. coli citrate synthase is a hexamer, each subunit having a value of M_r of 46,800 ($\pm 1,000$). The studies using Ferguson plots support the validity of carrying out SDS-polyacrylamide gel electrophoresis in the continuous system at pH 6.7, and cast doubts on the reliability of the discontinuous Tris-glycine method at pH 8.9.

Since this work was completed, the gene coding for E. coli citrate synthase (known as glt A) has been sequenced (Ner et al., 1983) in its entirety. Assuming that little or no post-translational modification occurs, the sequence of bases corresponds to a polypeptide

Figure 9. Densitometer Traces of Cross-Linked Citrate Synthase from
E. coli CA244 after SDS-Polyacryl-
amide Gel Electrophoresis



Enzyme cross-linked with (a) dimethylsuberimide and (b) glutaraldehyde as described in Methods.

chain of 427 amino acid residues and an M_r value of 48,069. This figure is in very close agreement with that observed by SDS-polyacrylamide gel electrophoresis at pH 6.7.

The cross-linking studies with dimethylsuberimidate confirm the findings of Tong and Duckworth (1975). They used the same reagent and suggested that E. coli citrate synthase is hexameric. The cross-linking studies using glutaraldehyde not only suggested the enzyme was hexameric but may also have yielded information regarding the arrangement of subunits. The preponderance of dimer, tetramer and hexamer suggests that dimer-dimer interactions are favoured more than say, trimer-trimer. Cross-linking patterns similar to these were found by Carpenter and Harrington (1972) with the hexameric enzyme leucine amino peptidase from bovine lens. They concluded that this enzyme was arranged as a trimer of dimers. These conclusions assume that the ability of neighbouring subunits to undergo cross-linking reflects their spatial arrangement in the hexameric native enzyme.

Under certain conditions, E. coli citrate synthase has been shown to dissociate into a mixture of hexamers and dimers. Tong and Duckworth (1975) observed that in the presence of 50mM KCl the enzyme existed as both a hexameric and dimeric species as analysed by ultracentrifugation studies. Increasing the KCl concentration to 100mM converted all the enzyme to hexamer. At pH 9.0 however, in the absence of KCl, only the dimer was detected. These results also suggest that the fundamental building block of the hexameric E. coli citrate synthase is the dimer.

As to the arrangement of the subunits there are three possible arrangements for a hexameric protein (figure 10). The various

interacting domains of the subunits are designated p, q, r and s, using the nomenclature of Cornish-Bowden and Koshland (1971). Arrangement (a) is the planar hexagon which has all isologous interactions between subunits, 3xp-p and 3xq-q with, say, p-p interactions more favourably cross-linked than q-q; (b) is the trigonal prism with two types of interaction, one heterologous, 6xp-q, and the other isologous, 3xr-r; (c) is an octahedral structure in which each subunit interacts with four others producing three types of interaction, one heterologous, 6xp-q, and two isologous, 3xr-r and 3xs-s. Electron microscopy studies of citrate synthase from both E. coli (Danson, 1974) and Acinetobacter lwoffii (Rowe and Weitzman, 1969) do not reveal any planar hexagon structures and on this evidence arrangement (a) would seem to be unlikely. Of the two remaining alternatives, (b) and (c), both can be explained from the cross-linking patterns observed. In the case of (b), the trigonal prism, the isologous r-r interactions would be more favoured than the heterologous p-q type. If p-q was more favoured than r-r a preponderance of trimer and hexamer would have resulted. Arrangement (c), the octahedral structure, would have either of the isologous interactions (r-r and s-s) preferentially cross-linked in order to produce the observed pattern.

'Large' citrate synthases from other Gram-negative organisms have also been observed to exhibit cross-linking patterns indicative of hexamers. The enzymes studied were from Acinetobacter calcoaceticus (Mitchell and Weitzman, 1983) and Pseudomonas aeruginosa (C.G. Mitchell, personal communication).

Since this work was completed, X-ray crystallographic studies on E. coli citrate synthase have been reported (Rubin et al., 1983).

These were performed at 15\AA resolution and indicate that the enzyme is composed of 4 subunits giving a total enzyme M_r value of 188,000. These conclusions were based on 24 tetrameric molecules occupying the unit cell and estimates of V_M (the molecular volume) suggest that two tetrameric units occupy the asymmetric unit. However, a resolution of 15\AA is low and more detailed information has been provided at resolutions of 2.7\AA and 1.7\AA in a crystallographic study of citrate synthase from pig heart and chicken heart (Remington et al., 1982). Further observations at higher resolution may resolve this discrepancy.

CHAPTER 2

The Citrate Synthase from *E. coli*

JA200/pLC26-17

The Citrate Synthase from *E. coli* JA200/pLC26-17

Introduction

The organism *E. coli* JA200/pLC26-17 contains several copies of a Col E1 - *E. coli* hybrid plasmid designated pLC26-17 in which the *E. coli* citrate synthase gene, glt A, has been incorporated (Guest, 1981). Guest also reported that this particular strain contained amplified levels of citrate synthase activity (approximately 5-fold the level found in *E. coli* JA200 - the strain lacking the hybrid plasmid). Should this increase in citrate synthase activity be represented in terms of enzyme protein, a means has thus been made available for the production of larger quantities of enzyme for structural studies. In addition, this technique would be particularly useful for studying citrate synthase from a mutant gene which produced an enzyme with relatively low specific activity.

Although Guest (1981) showed that *E. coli* JA200/pLC26-17 produced a functional citrate synthase, no work was carried out to determine if the enzyme retained the structural and regulatory properties of the wild-type *E. coli* citrate synthase. In the light of the observations made by Harford and Weitzman (1978) and Danson *et al.* (1979b), that a minor genetic change can bring about the production of a citrate synthase with greatly altered properties, it would seem necessary to verify that the glt A gene product of pLC26-17 was a wild-type *E. coli* citrate synthase. This finding would carry significance were this gene to be subsequently sequenced or used for large-scale production of *E. coli* citrate synthase.

Results

Citrate Synthase Assay

The enzyme was assayed spectrophotometrically at 412nm in 'Tris buffer A' as described for wild-type citrate synthase from E. coli CA244.

Enzyme Purification

An overnight culture (16h) grown aerobically in L broth (5l) at 37°C yielded 30g of cells. These cells were harvested as described in Methods. Following cell disruption and protamine sulphate treatment, the resultant cell-free extract was applied to a column of DEAE-Sephacel (30cmx3cm) which had previously been equilibrated with 'Tris buffer A'. The column was washed in the same buffer until no further protein was eluted (approximately 200ml). A linear gradient of 0.1M - 0.5M KCl was applied in 500ml of 'Tris buffer A'. Fractions (6.2ml) were collected at a flow rate of 25ml/h. Citrate synthase activity was detected in those fractions containing approximately 0.25M KCl. Fractions containing the enzyme at a specific activity greater than 17 units/mg were pooled and diluted 1:3 in 20mM Tris/HCl buffer, pH 8.0 containing 1mM EDTA. A Matrex Gel Red A column (25cmx1.3cm) was equilibrated with 20mM Tris/HCl pH8.0 containing 1mM EDTA and 50mM KCl. Following application of the enzyme extract, the column was washed with 70ml of this buffer before a linear gradient of 0 - 0.5M NaCl was applied in 200ml of the same buffer. Fractions (2ml) were collected at a flow rate of 10ml/h. Citrate synthase activity was detected in those fractions containing

approximately 50mM NaCl. Fractions containing the enzyme at a specific activity greater than 40 units/mg were found to behave as a single molecular species when analysed by SDS-polyacrylamide gel electrophoresis (figure 11), gel filtration in 6M guanidinium chloride and analytical ultracentrifugation in 'Tris buffer A'.

A summary of the 4-step purification procedure is shown in table 2. This represents a 40-fold increase in specific activity and a yield of 32mg of pure enzyme was obtained from 30g of cells.

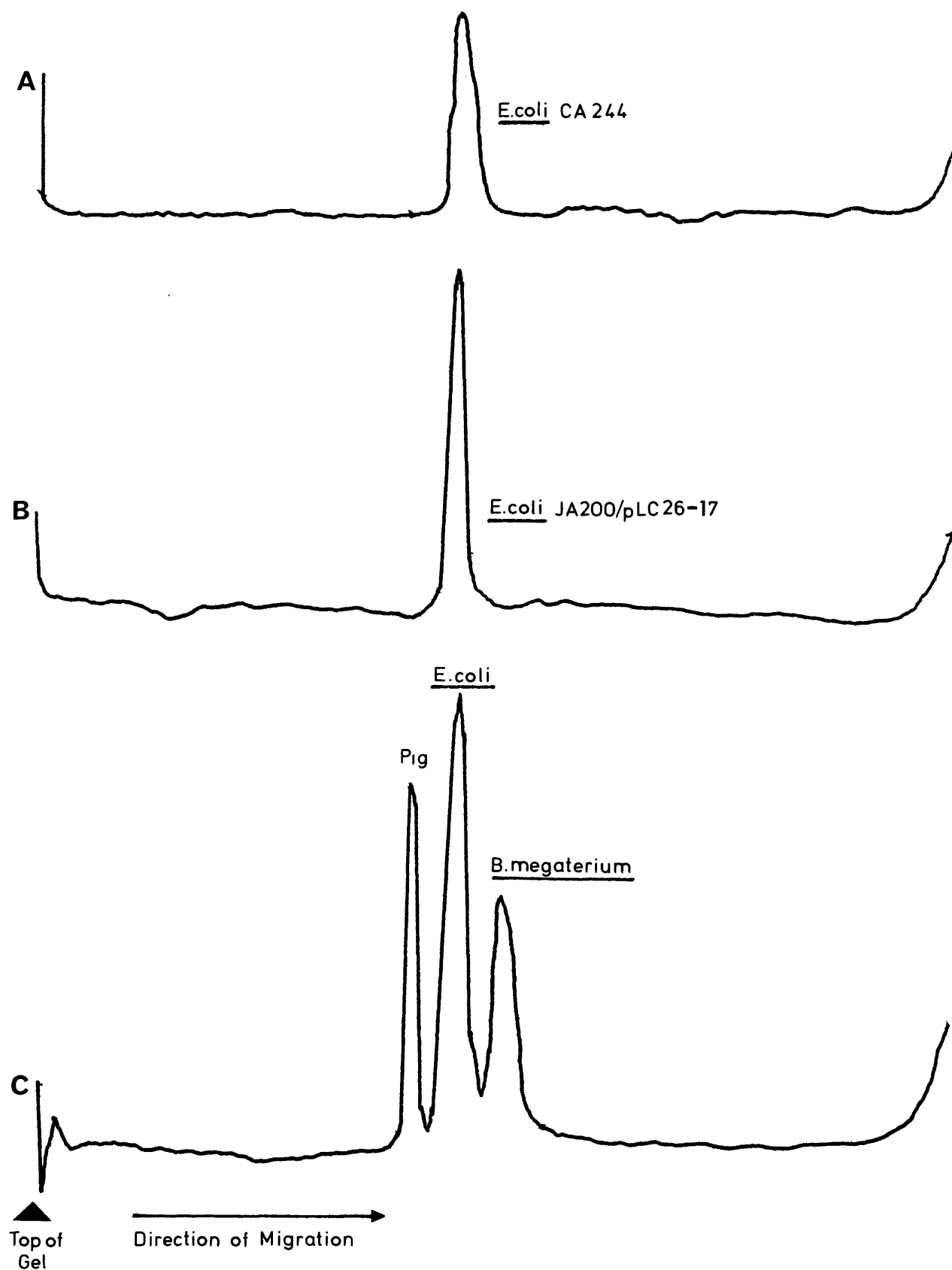
Determination of the M_r Value of the Native Enzyme

(a) Sedimentation-Equilibrium Ultracentrifugation

High-speed sedimentation-equilibrium studies were performed as described in Methods, with the enzyme in 'Tris buffer A'. A plot of $\log_n c$ (where c is the enzyme concentration) versus r^2 (r is the distance in cm from the centre of rotation) yielded a straight line after centrifugation for 120h at a rotor speed of 9975 r.p.m. The results are presented in figure 12. The linear relationship between $\log_n c$ and r^2 indicates the presence of a singular molecular species and the gradient, $d\log_n c/dr^2$ was determined. Assuming the amino acid composition of E. coli JA200 citrate synthase to be similar to that of E. coli CA244 a value for \bar{v} (partial specific volume) of 0.73 was used (Danson and Weitzman, 1973). From a series of weighings of known volumes, the density (ρ) of 'Tris buffer A' at 20°C was determined as 1.003 (± 0.0001) g/ml. The M_r value was calculated from the following equation (Bowen, 1970c):

$$M_r = \frac{2RT}{\omega^2(1 - \bar{v}\rho)} \cdot \frac{d\log_n c}{dr^2}$$

Figure 11. Densitometer Traces of Various Purified Citrate Synthases
after SDS-Polyacrylamide Gel Electrophoresis



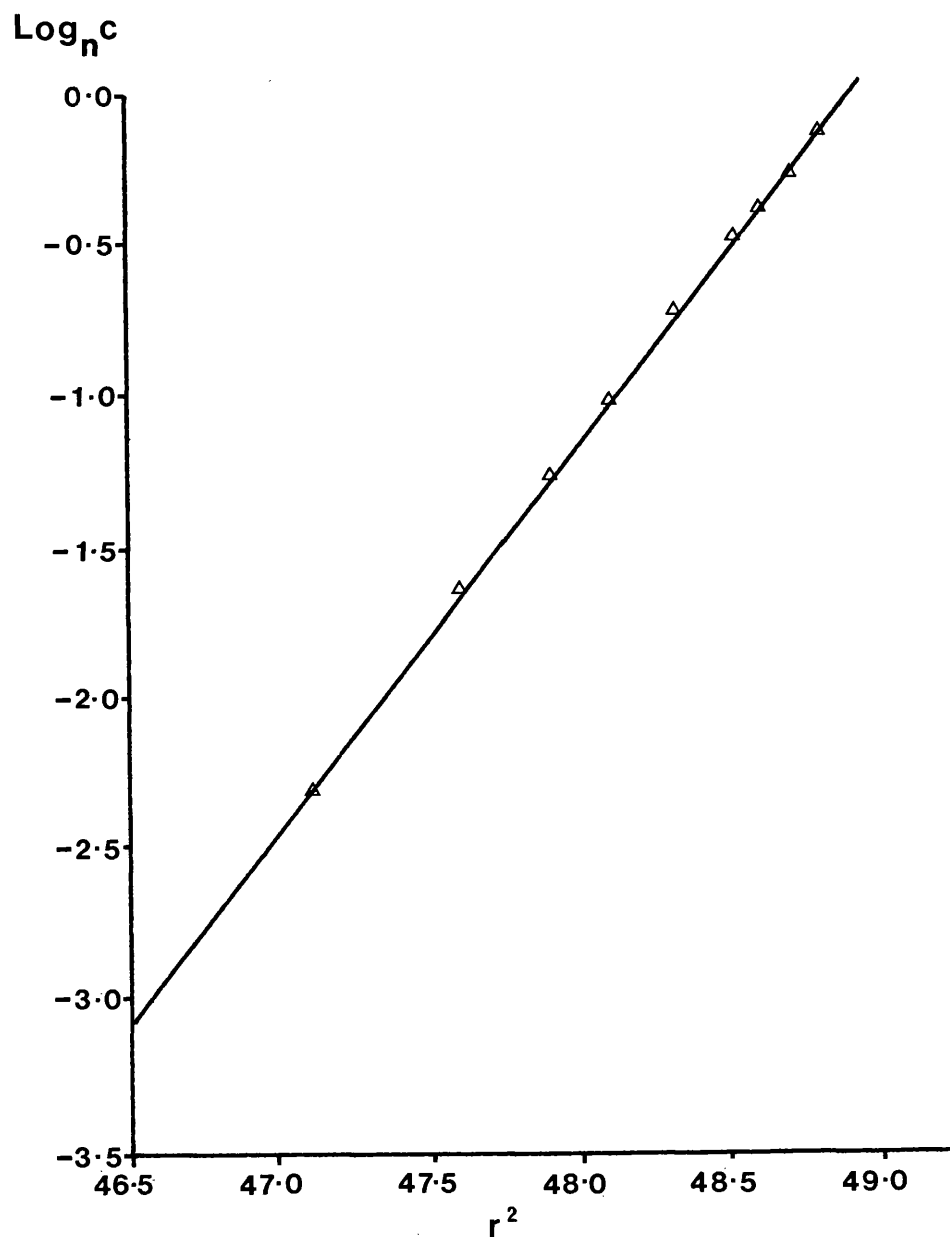
Electrophoresis at pH6.7 and staining were performed as described in Methods. (A) Chromosomally-coded citrate synthase from *E. coli* CA244; (B) Plasmid-coded citrate synthase from *E. coli* JA200/pLC26-17; (C) The citrate synthase from pig heart, *E. coli* JA200/pLC26-17 and *B. megaterium* separated on the same polyacrylamide gel in the presence of SDS.

Table 2. Purification of Citrate Synthase from E. coli

JA200/pLC26-17

Step	Volume (ml)	Total citrate synthase (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
(1) French press extract	58	5544	3793	1.5	-
(2) Supernatant from protamine SO ₄ treatment	58	5288	3686	1.4	95
(3) Pooled, selected fractions from DEAE-Sephacel ion exchange	50	5000	270	18.5	90
(4) Pooled, selected fractions from Matrex Gel Red A -elution with NaCl	8	2003	32	62.6	36

Figure 12. Sedimentation-Equilibrium Analysis of Purified Citrate Synthase from E.coli JA200/pLC26-17 - Relationship between $\log_n c$ and r^2



Protein concentration was 1mg/ml in 'Tris buffer A'. Centrifugation was performed at 20°C at a rotor speed of 9975 r.p.m. c is the protein concentration (mg/ml) at r , the distance (cm) from the centre of rotation.

where R is the molar gas constant, T is the absolute temperature and ω is the angular velocity of rotation in rad/s. A value of M_r of 227,000 ($\pm 5,000$) was calculated.

(b) Sedimentation-Velocity Ultracentrifugation

An alternative method of determining M_r values is using a combination of the Stokes radius (a_0) of a protein and its sedimentation coefficient ($s_{20,w}$) (Danson et al., 1979b).

The Stokes radius of E. coli JA200/pLC26-17 citrate synthase was determined by zonal gel filtration on Sephadex G-200 as described in Methods using the following proteins as standards: E. coli β -galactosidase, bovine liver catalase, pig heart lactate dehydrogenase and pig heart malate dehydrogenase. β -galactosidase was assayed spectrophotometrically at 420nm (Lederberg, 1950); catalase was detected spectrophotometrically at 410nm; lactate dehydrogenase and malate dehydrogenase were assayed spectrophotometrically at 340nm (Bergmeyer, 1974a,b). Citrate synthase was eluted as a single peak and the results are presented as a plot of Stokes radius (a_0) versus elution volume (ml) (figure 13). A linear relationship existed between the Stokes radius and elution volume and the Stokes radius for citrate synthase was determined as 5.6 (± 0.1) nm.

Sedimentation-velocity analyses were performed as described in Methods in 'Tris buffer A' at 20°C using citrate synthase at concentrations of 0.4mg/ml - 1.0mg/ml. The rate of sedimentation of the boundary was measured from the scans obtained. A typical scan is shown in figure 14. Plots of $\log_n x$ versus t were made, where x is the distance (cm) of the boundary from the centre of rotation at time t (min), and is shown in figure 15. The values for $d\log_n x/dt$ were determined from the gradients. The sedimentation

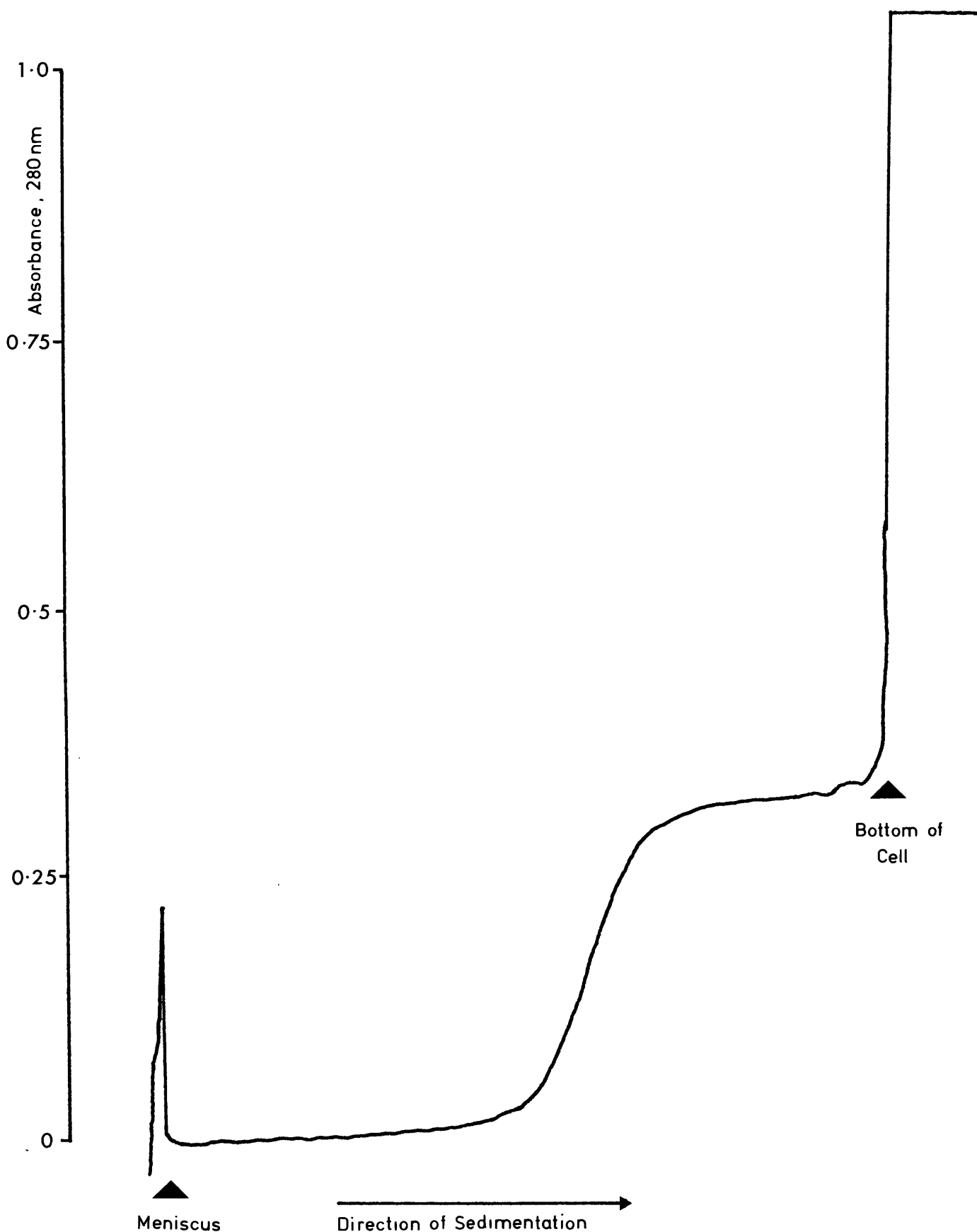
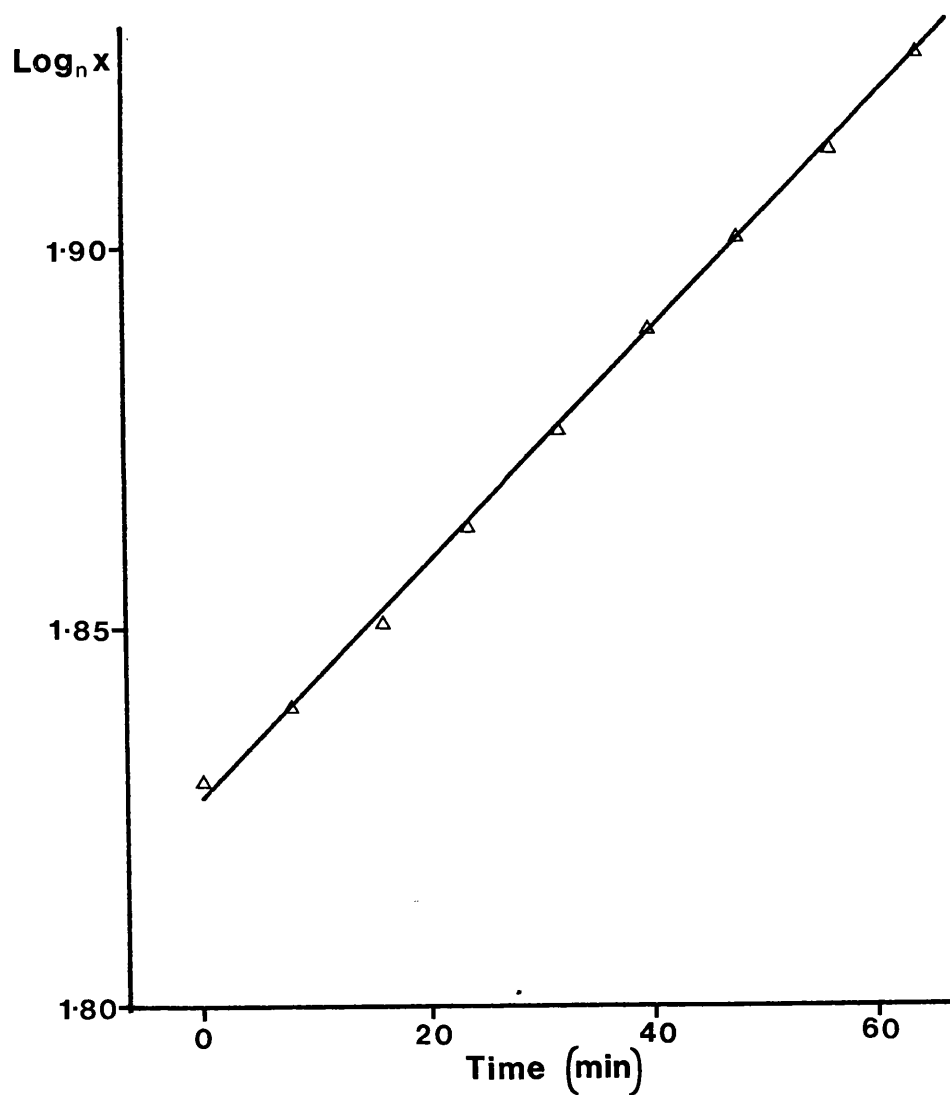


Figure 14. Sedimentation-Velocity Analytical Ultracentrifugation
of Purified Citrate Synthase from *E. coli* JA200/pLC26-17

Centrifugation in 'Tris buffer A' and absorbance scans at 280nm were performed as described in Methods. The scan shown is of a protein solution of concentration 0.4mg/ml at a rotor speed of 44,950 r.p.m.

Figure 15. Sedimentation-Velocity of Citrate Synthase from *E. coli*
JA200/pLC26-17 - Relationship between $\log_n x$ and Time (min)



Sedimentation-velocity analyses were performed in 'Tris buffer A' at 20°C as described in Methods. The figure shown represents a protein concentration of 0.4mg/ml and a rotor speed of 44,950 r.p.m. x is the distance (cm) of the protein boundary from the centre of rotation.

coefficient was calculated from the formula:

$$s = \frac{d \log_n x}{60 \omega^2 dt}$$

where ω is the angular velocity of the rotor (rads/s). Values for

$s_{20,w}$ were calculated from the formula:

$$s_{20,w} = \frac{s_{\text{obs.}} \eta_{\text{sol.}} (1 - \bar{v} \rho_{20,w})}{\eta_w (1 - \bar{v} \rho_{20,\text{sol.}})}$$

where $\eta_{\text{sol.}}$ and η_w are the viscosities of the solvent buffer and water respectively and $\rho_{20,\text{sol.}}$ and $\rho_{20,w}$ are the densities of solvent buffer and water respectively at 20°C. $s_{20,w}$ values were determined at various protein concentrations and the $s_{20,w}^0$ value was determined to be 11.5 (± 0.3) S by extrapolating to zero protein concentration (figure 16).

The M_r value for citrate synthase was calculated from the Stokes radius (a_0) and the value of $s_{20,w}^0$ using a combination of Stokes law (equation 1) and the Svedberg relationship (equation 2):

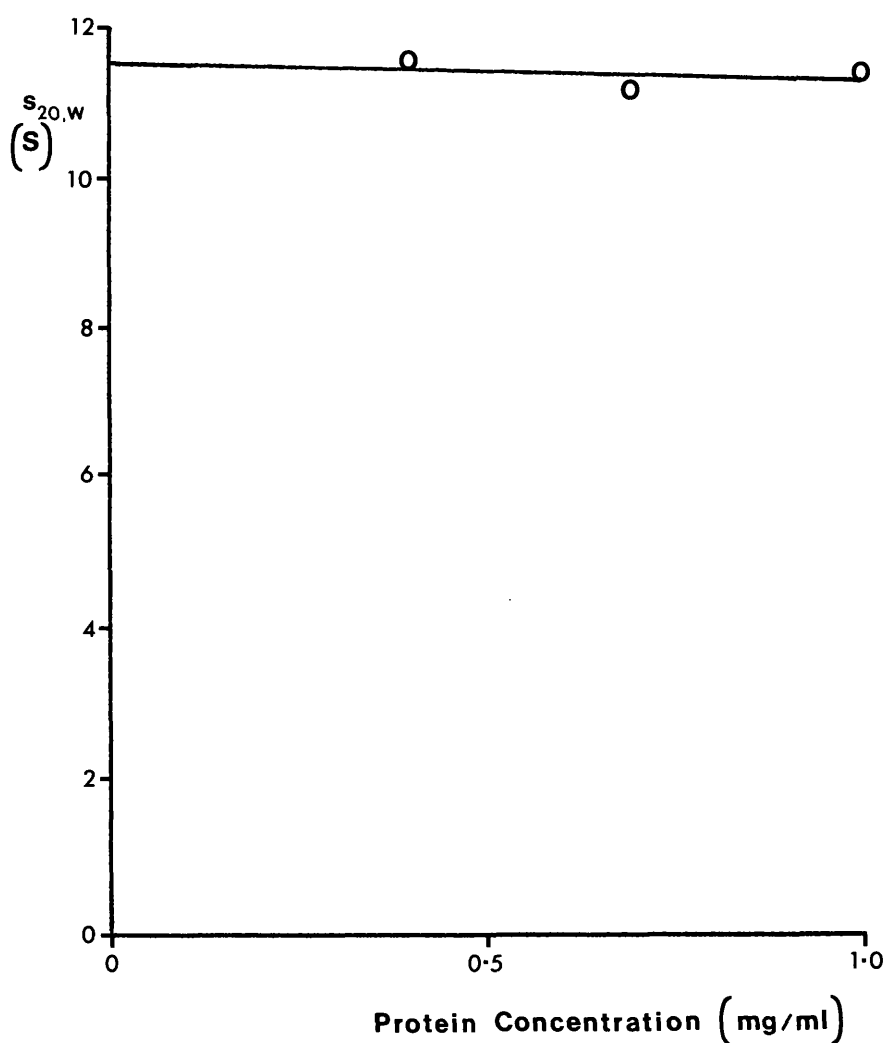
$$f = 6\pi\eta a_0 \quad (1)$$

$$M_r = \frac{RT s_{20,w}^0}{D(1 - \bar{v}\rho)} \quad (2)$$

where f = the frictional coefficient, η = solvent viscosity, R = gas constant, D = diffusion coefficient ($RT/N_A f$), N_A = Avogadro's number, \bar{v} = partial specific volume and ρ = solvent density. Combination of equations 1 and 2 gives the relationship:

Figure 16. Sedimentation-Velocity Analysis of E. coli JA200/pLC26-17

Citrate Synthase - Determination of $s_{20,w}^0$



E. coli JA200/pLC26-17 citrate synthase was subjected to sedimentation-velocity ultracentrifugation in 'Tris buffer A' as described in the text. The $s_{20,w}^0$ value was calculated by extrapolation to zero protein concentration.

$$M_r = \frac{6\pi\eta a_0 s_{20,w}^0 N_A}{(1 - \bar{v}\rho)}$$

From this equation a value for M_r was determined as 269,000 ($\pm 6,000$).

Determination of the M_r Value of the Subunit

(a) SDS-Polyacrylamide Gel Electrophoresis

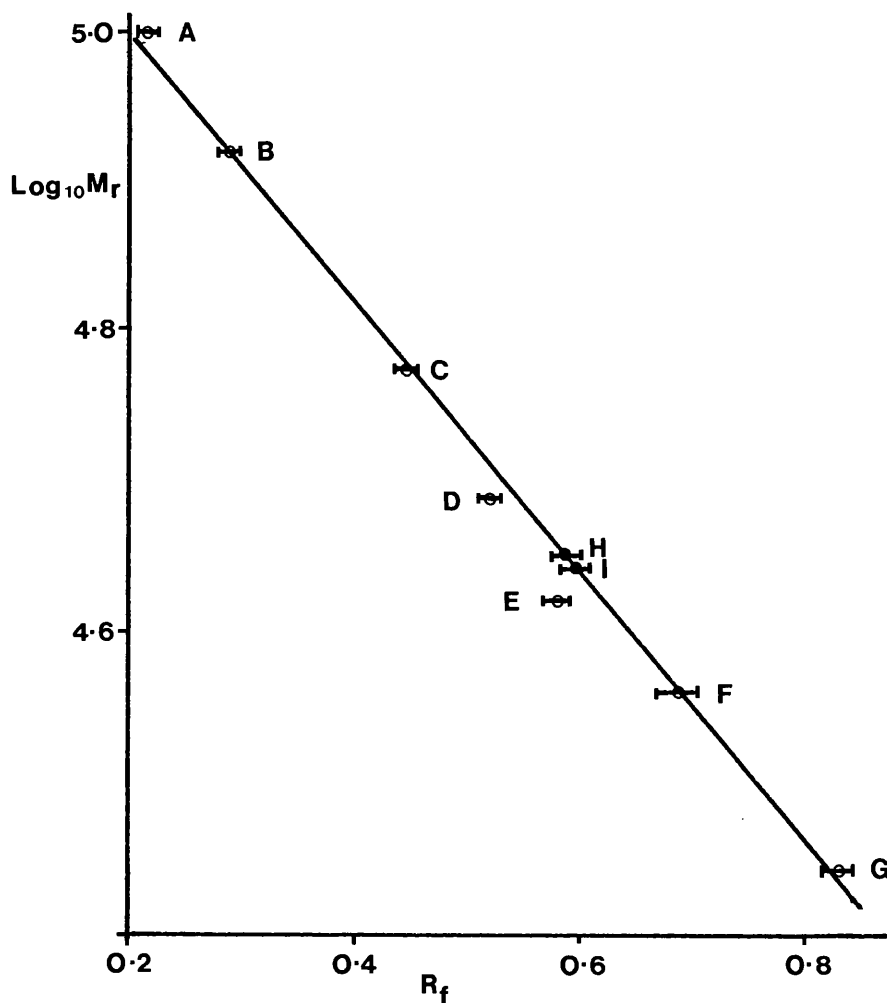
Purified citrate synthase from E. coli JA200/pLC26-17 was subjected to SDS-polyacrylamide gel electrophoresis at pH6.7 as described in Methods. The following proteins were also run as standards and their relative mobilities measured with respect to marker dye: E. coli pyruvate decarboxylase, E. coli lipoate acetyltransferase, E. coli dihydrolipoamide dehydrogenase, pig heart citrate synthase, rabbit muscle fructose biphosphate aldolase, pig heart lactate dehydrogenase and yeast triose phosphate isomerase. The gel scan (figure 11) of E. coli citrate synthase shows one molecular species only. The results plotted as $\log_{10} M_r$ versus relative mobility (Rf) are shown in figure 17. The citrate synthase from E. coli JA200/pLC26-17 ran with a relative mobility of 0.62 (± 0.01) corresponding to an M_r value of 44,000 ($\pm 2,500$).

(b) Gel Filtration in 6M Guanidinium Chloride

A column of Sephacryl S-300 (76cmx1.6cm) was prepared and run as described in Methods. Proteins used as standards were: horse heart cytochrome c, rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, rabbit muscle fructose biphosphate aldolase, bovine serum albumin, rabbit muscle phosphorylase a and E. coli β -galactosidase. The data were plotted in the form $\log_{10} 100Kd$ (where Kd is the distribution coefficient of the polypeptide) versus $N^{2/3}$ where N is the number of amino acid residues per polypeptide chain (Rydén, 1971).

Figure 17. SDS-Polyacrylamide Gel Electrophoresis at pH6.7 -

Relationship between $\log_{10} M_r$ and Relative Mobility (R_f)



SDS-polyacrylamide gel electrophoresis was performed at pH6.7 using the continuous system described in Method 1. Proteins used as standards were: (A) *E. coli* pyruvate decarboxylase (M_r 100,000); (B), *E. coli* lipoate acetyltransferase (M_r 83,000); (C) *E. coli* lipoamide dehydrogenase (M_r 56,500); (D), pig heart citrate synthase (M_r 49,000); (E), rabbit muscle fructose bisphosphate aldolase (M_r 42,000); (F), pig heart lactate dehydrogenase (M_r 36,000); and (G), yeast triose phosphate isomerase (M_r 28,000). (H) and (I) represent the citrate synthase from *E. coli* JA200/pLC26-17 and *B. megaterium* respectively. The bars represent the S.E.M. of the mobilities, relative to marker dye, of each protein in three separate determinations.

For each polypeptide K_d was calculated as described by Belew et al. (1978) from the following equation:

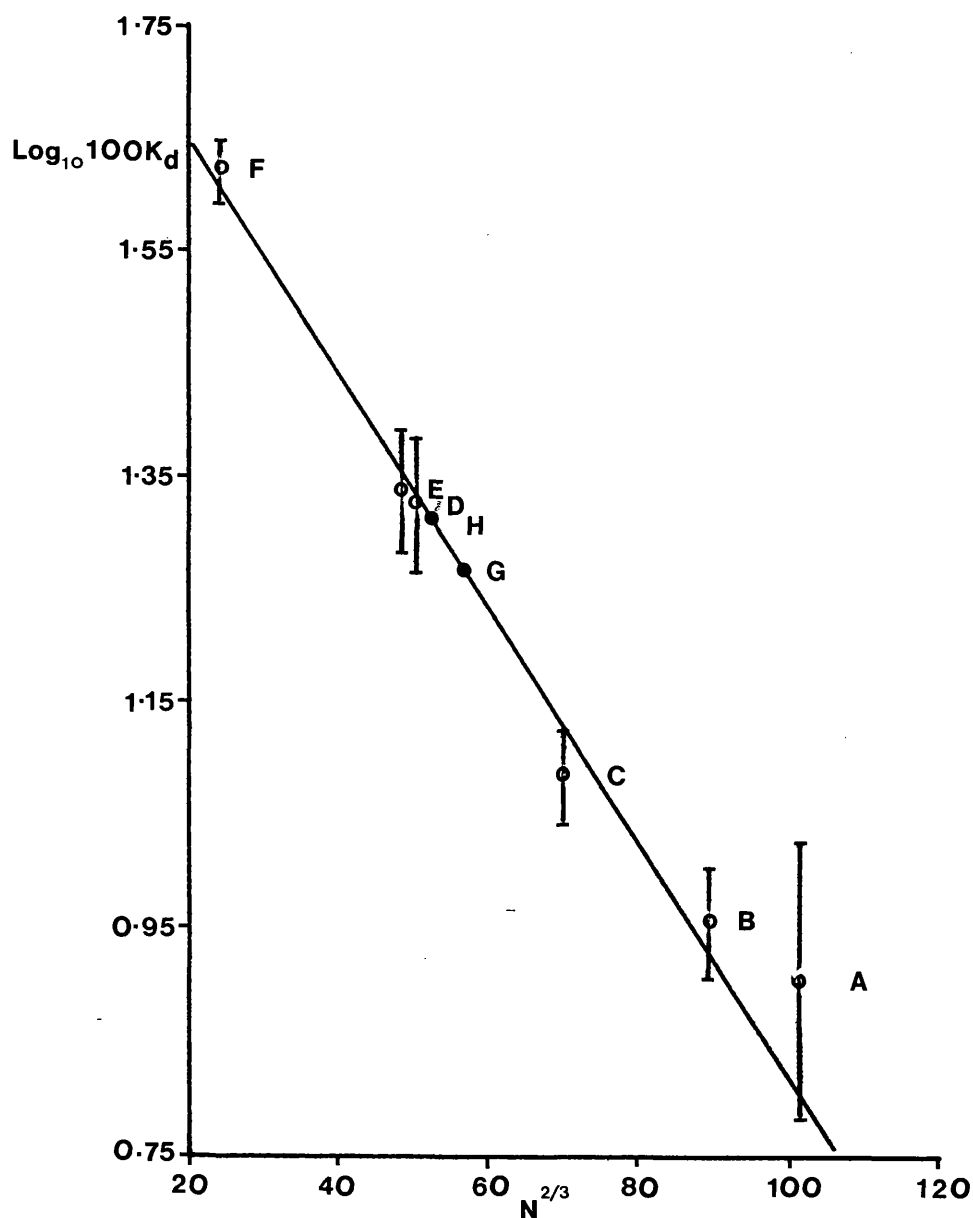
$$K_d = \frac{(V_e - V_o)}{(V_{re} - V_o)}$$

where V_e is the elution volume of the polypeptide and V_{re} and V_o are the total and void volumes respectively of the column determined as described in the Methods. The data are presented in figure 18. Citrate synthase was eluted as a single peak. A K_d value of 0.185 (± 0.006) was obtained corresponding to a polypeptide chain length of 420 (± 44) amino acids. From the amino acid composition of E. coli CA244 citrate synthase (Danson and Weitzman, 1973), and once again assuming reasonable similarity between the two enzymes, a value of M_r for the subunit of E. coli JA200/pLC26-17 citrate synthase was determined as 48,000 ($\pm 5,000$).

Determination of Subunit Number

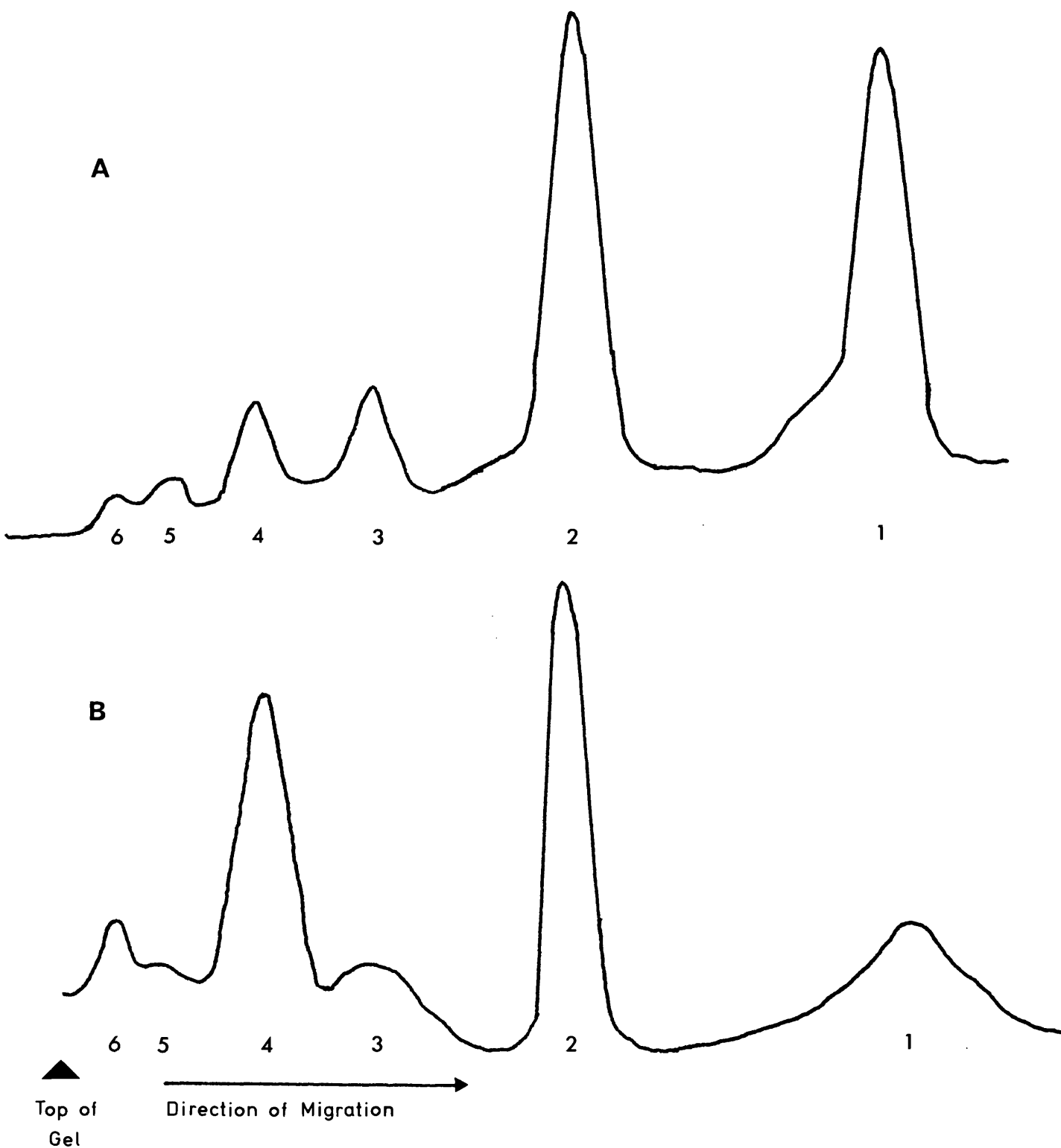
From the values of M_r determined for the native enzyme (230,000 - 270,000) and the subunit (44,000 - 48,000) it would appear that the citrate synthase from E. coli JA200/pLC26-17 is made up of 4.8 - 6.1 subunits, that is 5 - 6. To determine the number of subunits cross-linking studies were carried out using either dimethylsuberimidate or glutaraldehyde as described for E. coli CA244. As before, the cross-linked samples were analysed by SDS-polyacrylamide gel electrophoresis. The resulting gel scans are shown in figure 19. With each cross-linking reagent 6 bands were obtained, indicating E. coli JA200/pLC26-17 citrate synthase is hexameric. The distributions of the various cross-linked species,

Figure 18. Gel Filtration under Denaturing Conditions in 6M Guanidinium Chloride on Sephacryl S-300 - Relationship between $\log_{10} 100K_d$ and $N^{2/3}$



Gel filtration in 6M guanidinium chloride on Sephacryl S-300 was performed as described in Methods. N is the number of amino acids per polypeptide chain and K_d is the distribution coefficient calculated as described in the text. Proteins used as standards were: (A), *E. coli* β -galactosidase ($N=1021$); (B), rabbit muscle phosphorylase a ($N=841$); (C), bovine serum albumin ($N=579$); (D), rabbit muscle fructose bisphosphate aldolase ($N=358$); (E), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase ($N=330$); and (F), horse heart cytochrome c ($N=104$). (G) and (H) represent citrate synthase from *E. coli* JA200/pLC26-17 and *B. megaterium* respectively. The bars represent the S.E.M from five separate filtration experiments.

Figure 19. Densitometer Traces of Cross-Linked Citrate Synthase
from *E. coli* JA200/pLC26-17 after SDS-Polyacrylamide
Gel Electrophoresis



Enzyme was cross-linked with (A) dimethylsuberimide and (B) glutaraldehyde, as described in Methods.

were very similar to those obtained for E. coli CA244 (figure 9).

Kinetic and Regulatory Properties

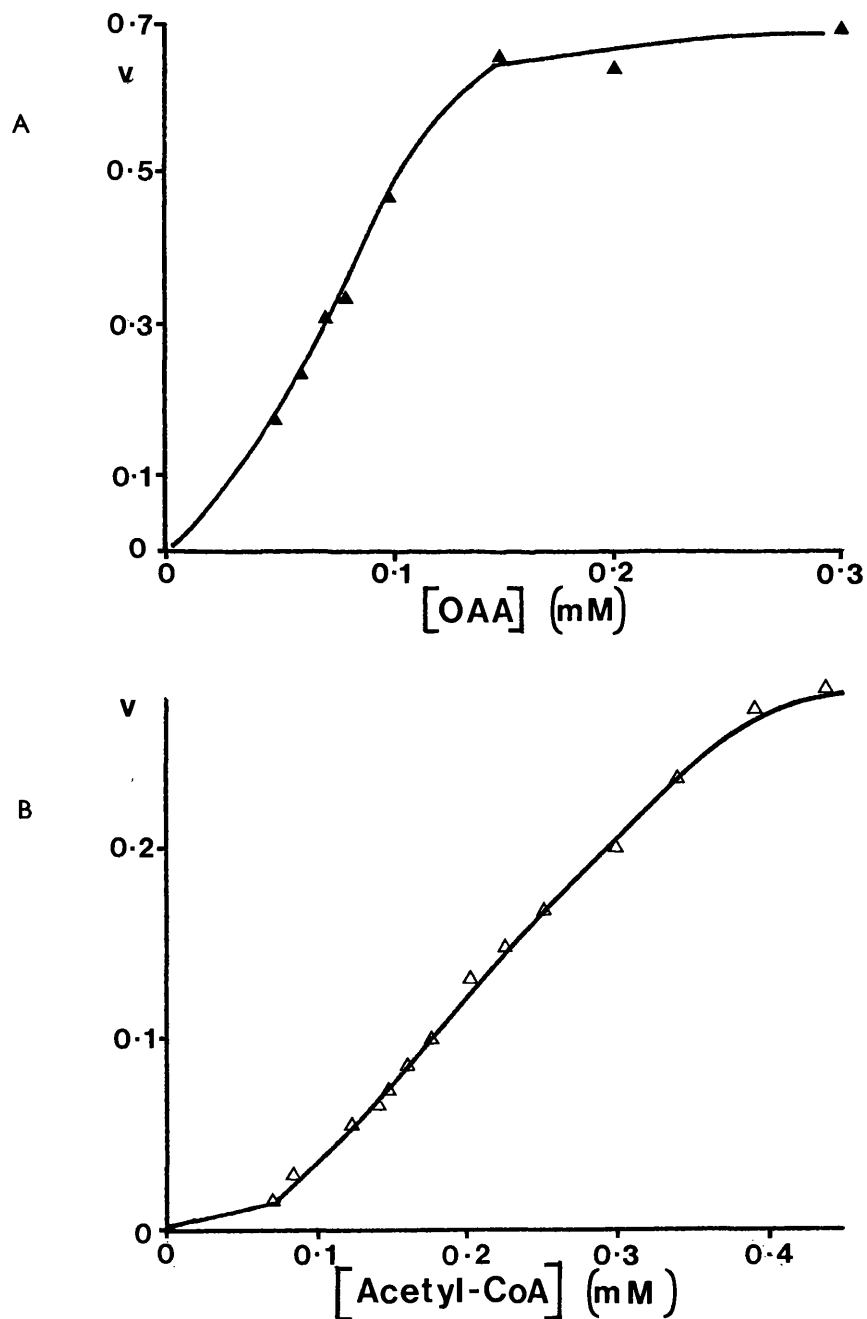
The kinetics of purified citrate synthase from E. coli JA200/pLC26-17 were analysed in 20mM Tris/HCl, pH8.0, containing 1mM EDTA. Plots of v (reaction velocity) versus $[S]$ (substrate concentration) for both acetyl-CoA and oxaloacetate (figure 20) were sigmoidal in nature. Plots of $[S]^2/v$ versus $[S]^2$ were found to be linear for both substrates (figure 21) and $S_{0.5}$ and apparent V_{max} values were determined for direct linear plots of V versus $[S]^2$. The $S_{0.5}$ for oxaloacetate was determined to be $77.5 (\pm 9.9) \mu M$ and the $S_{0.5}$ for acetyl-CoA was determined as $359 (\pm 22) \mu M$. Hill plots of $\log_{10} [S]$ versus $\frac{v}{v_{app} - v}$ were constructed (figure 22). A Hill coefficient (n) of $2.1 (\pm 0.1)$ was obtained for oxaloacetate and the corresponding value determined for acetyl-CoA was $2.0 (\pm 0.1)$. The values of the Hill coefficients for each substrate indicate co-operativity.

The inhibitory effects of 2-oxoglutarate and NADH were investigated in 20mM Tris/HCl, pH8.0 containing 1mM EDTA. The data were compared directly with those obtained using chromosomally-coded citrate synthase for E. coli CA244 under identical conditions (figure 23). No difference in the responses of the plasmid-coded and chromosomally-coded enzyme was detected.

Discussion

The plasmid-coded citrate synthase from E. coli JA200/pLC26-17 has been shown to have a value of M_r of 230,000 - 270,000, and possessed 6 subunits each having M_r values of 46,000 - 48,000.

Figure 20. Relationship of Reaction Rate (v) versus $[S]$ for
Citrate Synthase from *E. coli* JA200/pLC26-17



Enzyme assays were performed in 20mM Tris/HCl, pH8.0, 1mM EDTA at 412nm. v is measured in arbitrary units.
 (A) v versus $[OAA]$ at a fixed concentration of acetyl-CoA of 150 μ M.
 (B) v versus $[acetyl-CoA]$ at a fixed concentration of oxaloacetate of 200 μ M.

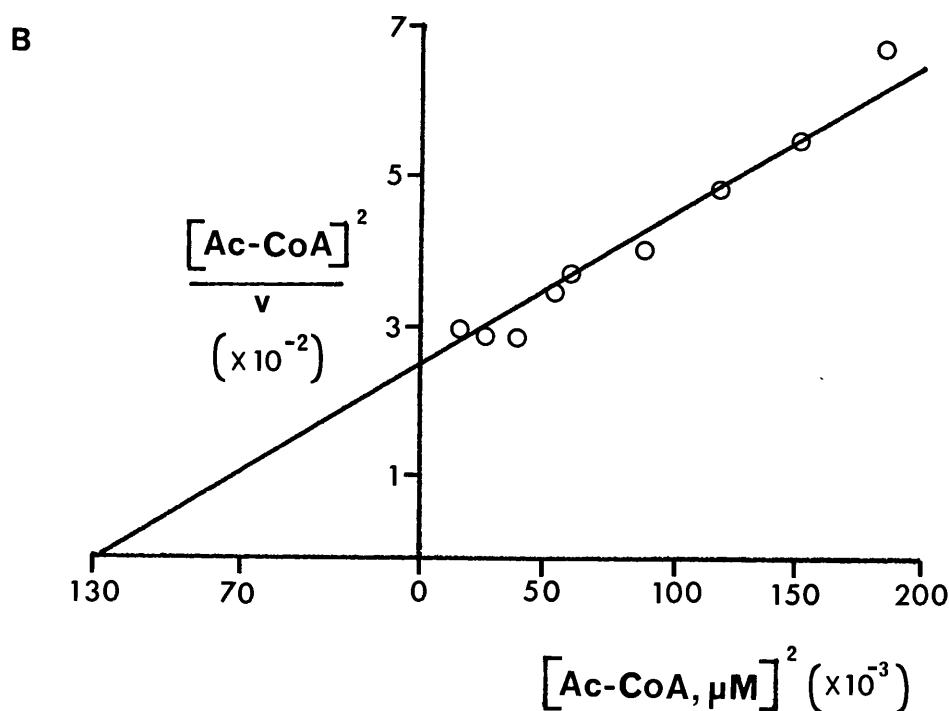
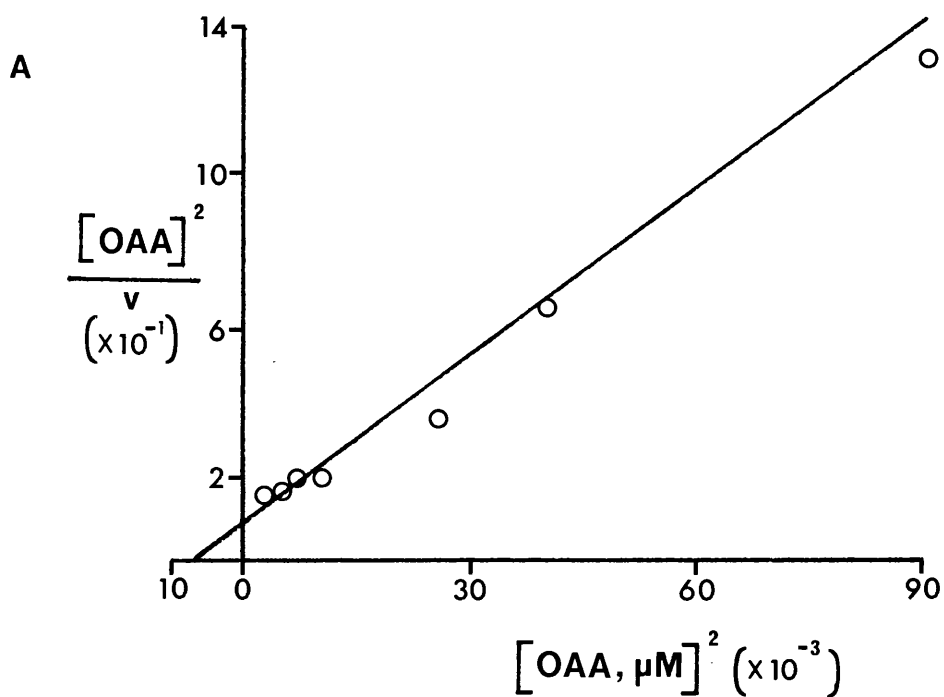


Figure 21. Plots of $[S]^2/v$ versus $[S]^2$ for Citrate Synthase from *E. coli* JA200/pLC26-17

Enzyme assays were performed in 20mM Tris/HCl, pH8.0, 1mM EDTA at 412nm.
 (A) Oxaloacetate in the presence of 150μM acetyl-CoA.
 (B) Acetyl-CoA in the presence of 200μM oxaloacetate.

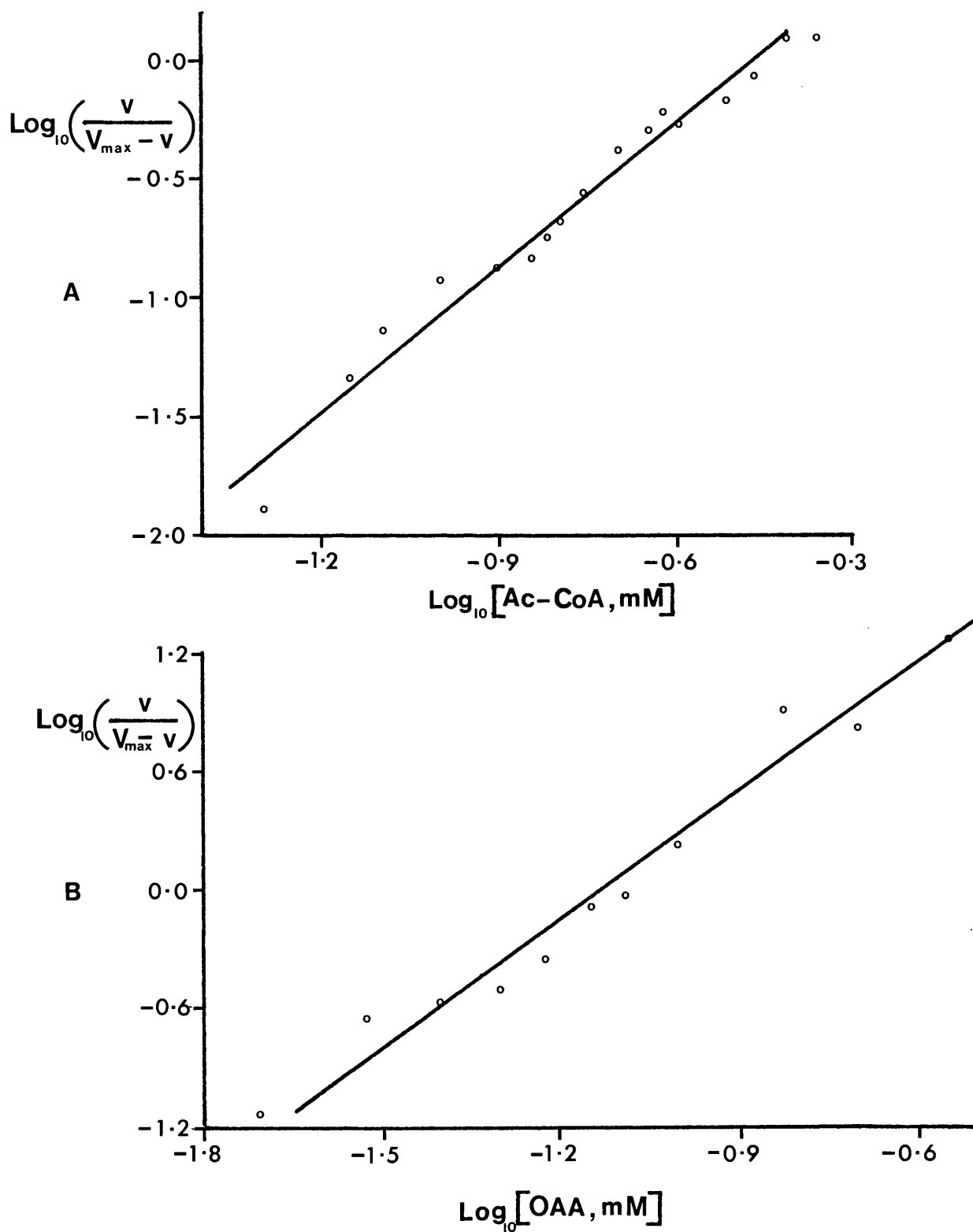
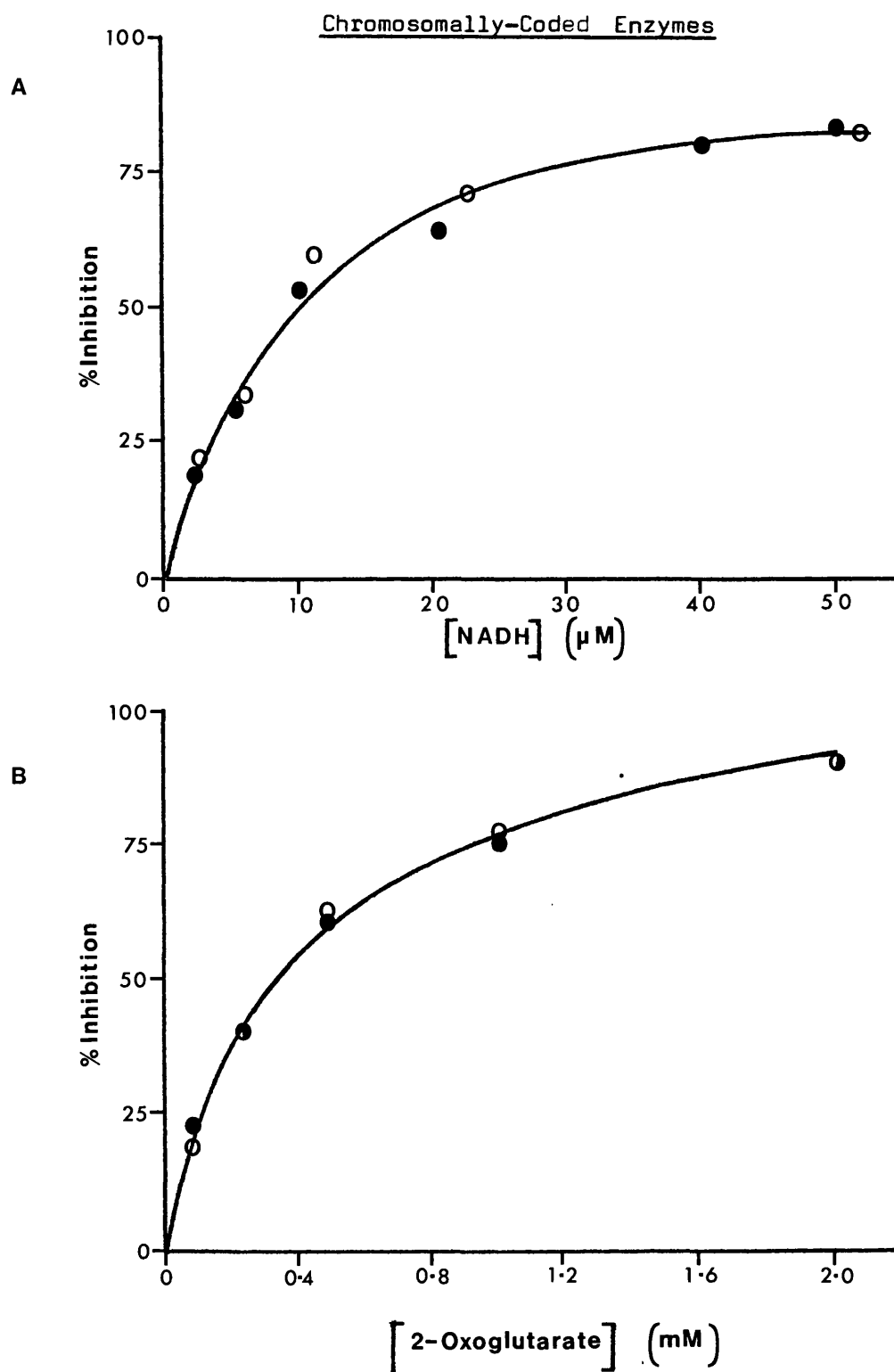


Figure 22. Hill Plots of $\log_{10}(v/V_{\max}-v)$ versus $\log_{10}[S]$ for Citrate Synthase from *E. coli* JA200/pLC26-17

The plots were constructed from the data obtained from figure 21.
 (A) Using acetyl-CoA and (B) using oxaloacetate.

Figure 23 Inhibition of *E. coli* Citrate Synthase by NADH and 2-Oxoglutarate - Comparison of Plasmid-Coded and



Inhibition of plasmid-coded (○) and chromosomally-coded (●) citrate synthase by NADH (A) and 2-oxoglutarate (B). Citrate synthase was assayed spectrophotometrically at 412nm using 150 μM acetyl-CoA and 50 μM oxaloacetate.

Both substrates showed sigmoidal dependences and the enzyme was inhibited to the same degree as chromosomally-coded citrate synthase by 2-oxoglutarate and NADH. A summary of the compared properties of the plasmid-coded and chromosomally-coded E. coli citrate synthases is shown in table 3. In all aspects studied, there is a very close resemblance between the two enzymes. It therefore appears that the cloning of the wild-type qlt A gene into a hybrid plasmid produces citrate synthase that is unaltered both structurally and kinetically. This is an important factor when considering the cloning of a mutant qlt A gene in order to produce greater quantities of a mutant citrate synthase. Clearly the technique would be unsatisfactory if, in the cloning of the mutant gene or the 'overproduction' of the mutant enzyme, an interesting characteristic property was lost or modified. Naturally, the results obtained in this instance do not preclude a possible alteration in the properties of a mutant enzyme should a mutant qlt A gene be successfully cloned.

When the purification procedure of citrate synthase from E. coli JA200/pLC26-17 is compared with that of E. coli CA244 (tables 2 and 1) it is apparent that not only is a shorter procedure possible with E. coli JA200/pLC26-17 but that 32mg of pure plasmid-coded enzyme are obtained compared with only 0.8mg of pure chromosomally-coded citrate synthase from the same weight of cells (30g).

Large-scale production of citrate synthase from a strain of E. coli containing a cloned qlt A gene has also been reported by Duckworth and Bell (1982). In this case a drug-resistance plasmid containing the entire E. coli qlt A gene was introduced into a strain of E. coli and produced in multiple copies. Citrate synthase was

Table 3. Comparison of the Properties of Chromosomally-Coded
and Plasmid-Coded Citrate Synthase from *E. coli*

	<u><i>E. coli</i> CA244</u> (chromosomally-coded)	<u><i>E. coli</i> JA200/pLC26-17</u> (plasmid-coded)
Enzyme M_r	230,000 - 270,000 ^{a,b,c}	230,000 - 270,000
Subunit M_r	47,000	46,000 - 48,000
No. of subunits	6	6
Substrate Dependence:		
Oxaloacetate	sigmoidal ^d	sigmoidal
Acetyl-CoA	sigmoidal ^d	sigmoidal
Oxaloacetate, $S_{0.5}$ (μ m)	54 ^d	78
Acetyl-CoA, $S_{0.5}$ (μ m)	390 ^d	359
Hill coefficient, OAA	1.8 ^d	2.1
Hill coefficient, Ac-CoA	1.9 ^d	2.0
Inhibition by 50 μ m NADH (%)	80	81
Inhibition by 1mM 2-oxo- glutarate (%)	79	77

(a), Wright and Sanwal (1971); (b) Danson and Weitzman (1973);

(c), Tong and Duckworth (1975); (d), Danson (1974).

then produced to such an extent that the enzyme represented approximately 8% of the soluble protein of the cells. Approximately 80-100g of the cells yielded 600-800mg of enzyme. Duckworth and Bell (1982) also showed that this enzyme was identical to that coded chromosomally in terms of the 16 N-terminus amino acids, subunit M_r value and inhibition by NADH.

A technique exists for increasing the plasmid copy number of pLC26-17 by treatment of E. coli JA200/pLC26-17 with sublethal concentrations of chloramphenicol (Robinson et al., 1983). Cell division and chromosomal DNA replication were inhibited but plasmid replication continued until the plasmid copy number increased from approximately 7 to 300 copies per cell. Unfortunately this was not reflected in the increase in specific activity of citrate synthase in cell-free extracts. This may be due to the control of expression of the qlt A gene and suggests that the promoter region of qlt A is also contained on this plasmid. A limit may exist for the levels of citrate synthase contained in E. coli above which the metabolism and growth of the cells may be detrimentally disturbed.

CHAPTER 3

The Citrate Synthase from Bacillus

megaterium

The Citrate Synthase from *Bacillus megaterium*

Introduction

Citrate synthase has been well characterised from a wide variety of eukaryotic sources. The enzyme is of the 'small' type with a M_r of approximately 100,000 (Weitzman and Danson, 1976) and is dimeric (Wu and Yang, 1970; Singh et al., 1970; Moriyama and Srere, 1971). Detailed kinetic studies have been performed (Matsuoka and Srere, 1973; Matlib et al., 1978; Kubicek and Röhr, 1980; Bayer et al., 1981) and information about the active site has been provided by x-ray crystallography (Wiegand et al., 1979). However, the citrate synthase from Gram-positive bacteria has been somewhat overlooked. The enzyme has also been shown to be of the 'small' type (Weitzman and Danson, 1976) and Higa and Cazzulo, (1976) observed the citrate synthase from *Bacillus stearothermophilus* to have a value of M_r of approximately 100,000. The enzyme is inhibited isosterically by ATP (Flechner and Hanson, 1969; Harford and Weitzman, 1975; Higa and Cazzulo, 1976) and displays hyperbolic substrate dependences. In its molecular and kinetic properties so far studied, citrate synthase from Gram-positive bacteria resembles the enzyme isolated from eukaryotes.

Unfortunately no detailed studies have been reported on either the subunit composition of the enzyme or the roles of particular amino acid side-chains in the catalytic process. Johnson and Hanson (1974) attempted unsuccessfully to purify the enzyme from *Bacillus subtilis*, and although Higa and Cazzulo (1976) reported purification

to homogeneity of Bacillus stearothermophilus citrate synthase, no detailed structural studies were performed.

Comparisons of structure-function relationships between 'large' and 'small' citrate synthases cannot be complete without more information about the 'small' enzyme from Gram-positive bacteria. It has been suggested that eukaryotic organelles, such as mitochondria, may have evolved endosymbiotically from prokaryotic origins. On the basis of this hypothesis it may be anticipated that evolutionary relationships are likely to be found between enzymes from prokaryotes and mitochondria. More detailed studies of the citrate synthase from Gram-positive bacteria may allow comparisons to be made with the enzyme from eukaryotic organisms at a finer level. Therefore, attempts were made to purify and characterise the citrate synthase from the Gram-positive bacterium Bacillus megaterium.

Results

Assay of Bacillus megaterium Citrate Synthase

The enzyme was assayed spectrophotometrically at 412nm in 'Tris buffer A' unless otherwise stated.

Stability of Bacillus megaterium Citrate Synthase

Early attempts at enzyme purification involved extracting the citrate synthase in 'Tris buffer A'. These proved unsuccessful, as the enzyme rapidly lost activity and was usually completely inactive within 24h. Johnson and Hanson (1974) reported that the stability of citrate synthase from crude preparations of B. subtilis

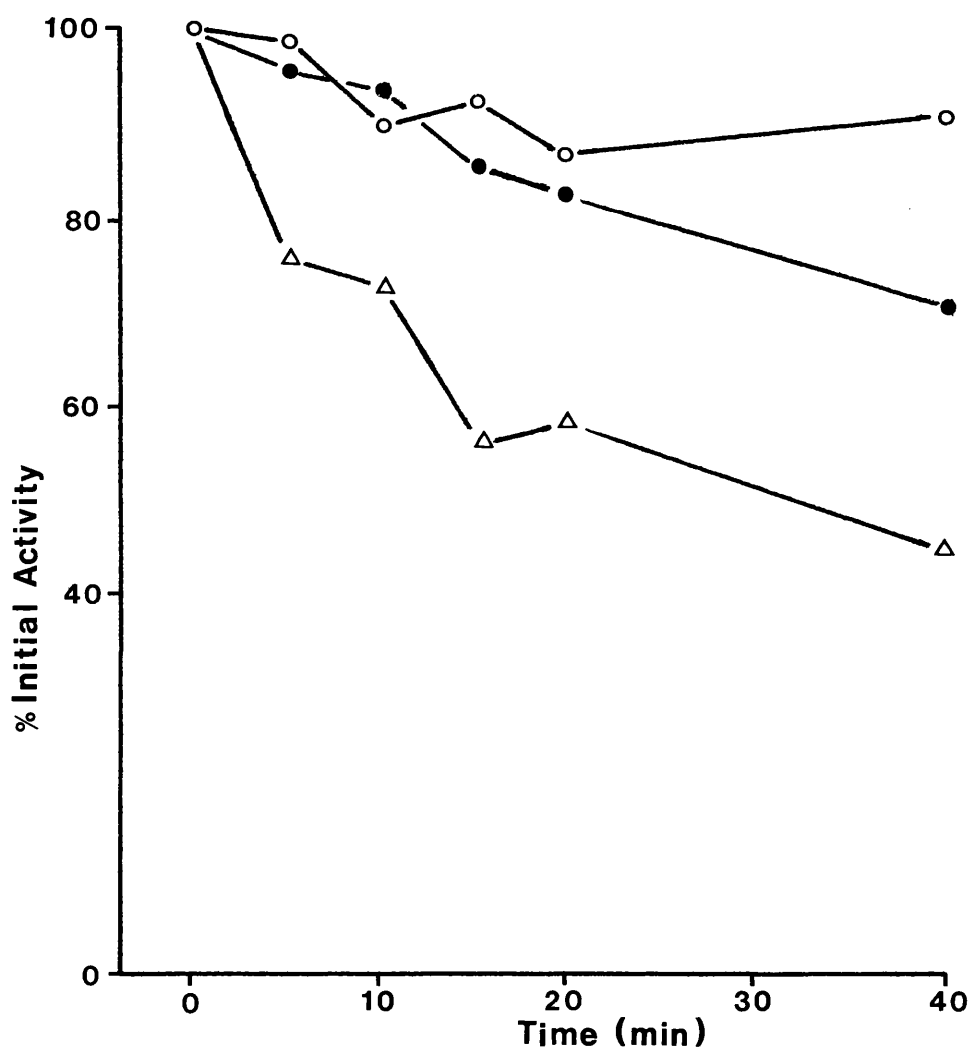
was greatly enhanced by the presence of glycerol. The stability of the enzyme from B. megaterium was investigated using crude preparations in buffers of varying composition and the results are presented in figure 24. These show that, of the buffers used at 25°C, greatest stability in terms of retained enzyme activity was achieved in the presence of both 100mM KCl and 20% (v/v) glycerol. Preparations were therefore carried out in 20mM Tris/HCl, pH8.0, containing 1mM EDTA, 100mM KCl and 20% (v/v) glycerol hereafter referred to as 'Tris buffer B'. As an added precaution against proteolysis, 0.15mM phenylmethanesulphonylfluoride (PMSF) was also included.

Enzyme Purification

Cells from 10l of an overnight (16h) culture in L broth were harvested and washed by resuspension in 200ml of 'Tris buffer B' containing 0.15mM PMSF. The cells were collected by centrifugation and the pellet (35g) was suspended in 100ml of 'Tris buffer B' containing 0.15mM PMSF and lysozyme (25mg/ml). The suspension was stirred for 2h. This and all subsequent steps were carried out at 4°C. The cells were then lysed using a French press.

After treatment with protamine sulphate, as described in Methods, the supernatant was applied to a column (30cmx3cm) of DEAE-Sephacel, previously equilibrated in 'Tris buffer B' containing 0.15mM PMSF. The column was then washed in 200ml of the same buffer before a linear gradient of 0.1M - 0.5M KCl was applied in 500ml of buffer. Fractions (5.2ml) were collected at a flow rate of 15ml/h. Citrate synthase activity was detected in

Figure 24. The Stability of Citrate Synthase from *B. megaterium*
in Various Buffers at 25°C - % Initial Activity Versus
Time



B. megaterium citrate synthase was incubated with the following buffers as described in the text: (O), 20mM Tris/HCl, pH8.0 containing 1mM EDTA, 100mM KCl and 20% (v/v) glycerol ('Tris buffer B'); (●), 20mM Tris/HCl, pH8.0 containing 1mM EDTA and 100mM KCl ('Tris buffer A'); and (Δ), 20mM Tris/HCl, pH8.0 containing 1mM EDTA ('Tris buffer C').

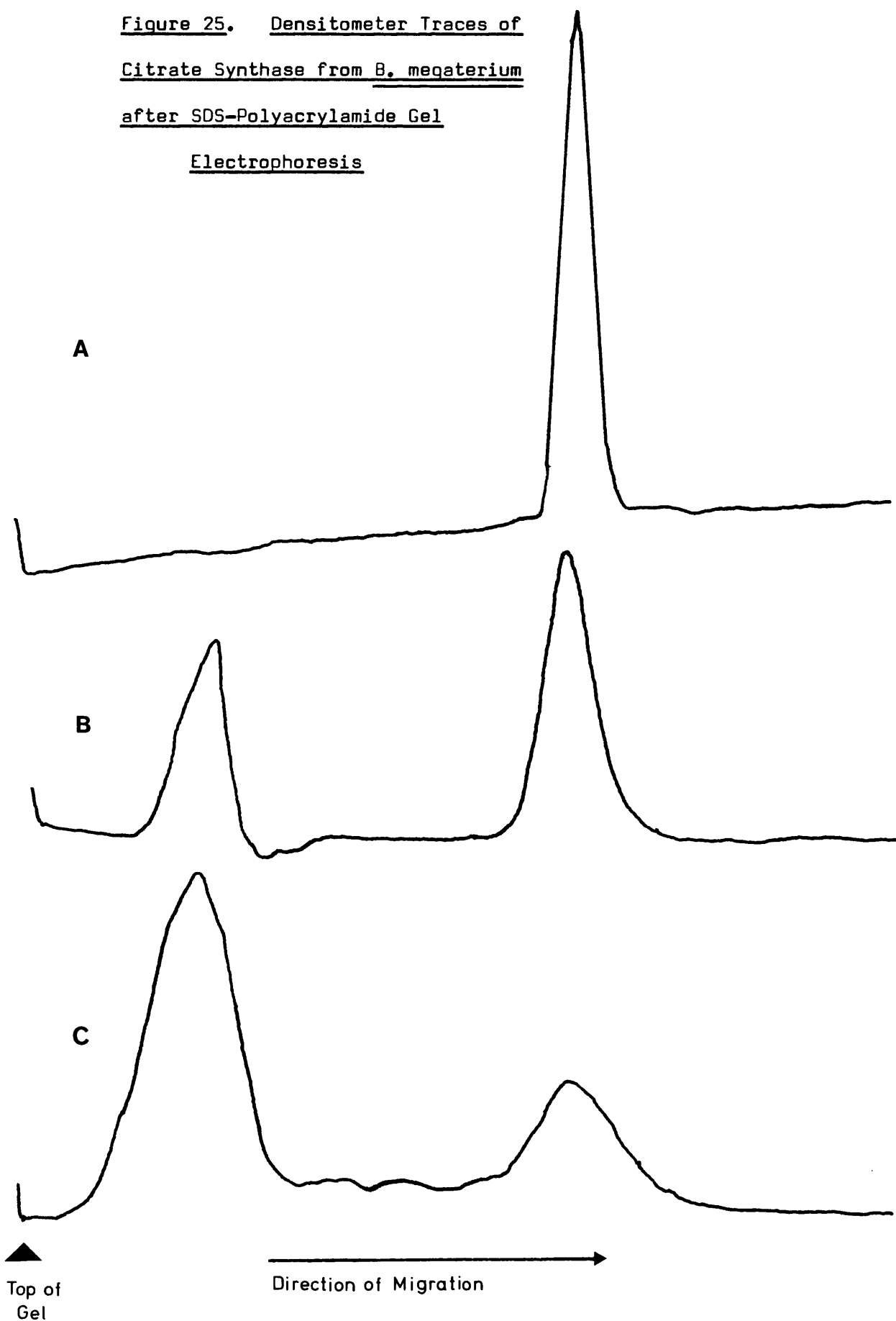
fractions containing approximately 0.4M KCl. Those fractions containing enzyme at a specific activity greater than 2 units/mg were pooled and dialysed for 3h against 2l of 'Tris buffer B' containing 50mM KCl instead of 100mM KCl.

A column (25cmx1.3cm) of Matrex Gel Red A was prepared and equilibrated with 'Tris buffer B' containing 50mM KCl instead of 100mM KCl. This buffer (20ml) was then used to wash the column after the application of the enzyme extract. A linear gradient of 0 - 0.5M NaCl was applied in 100ml of the wash buffer and fractions (1ml) were collected at a flow rate of 10ml/h. Citrate synthase activity was detected in fractions containing approximately 0.3M NaCl. Those fractions containing enzyme at a specific activity of greater than 35 units/mg were pooled and dialysed for 3h against 'Tris buffer B'.

The dialysed product was applied to a column (8cmx0.8cm) of Matrex Gel Red A, previously equilibrated with 'Tris buffer B'. Citrate synthase was eluted with this buffer containing 0.1mM oxaloacetate and 0.1mM acetyl-CoA. Fractions (1ml) were collected at a flow rate of 10ml/h and citrate synthase activity was detected after approximately 9ml. Those fractions containing the enzyme at a specific activity greater than 50 units/mg were analysed for purity by SDS-polyacrylamide gel electrophoresis and sedimentation-velocity ultracentrifugation and were found to behave as single molecular species in both systems (figures 25 and 26).

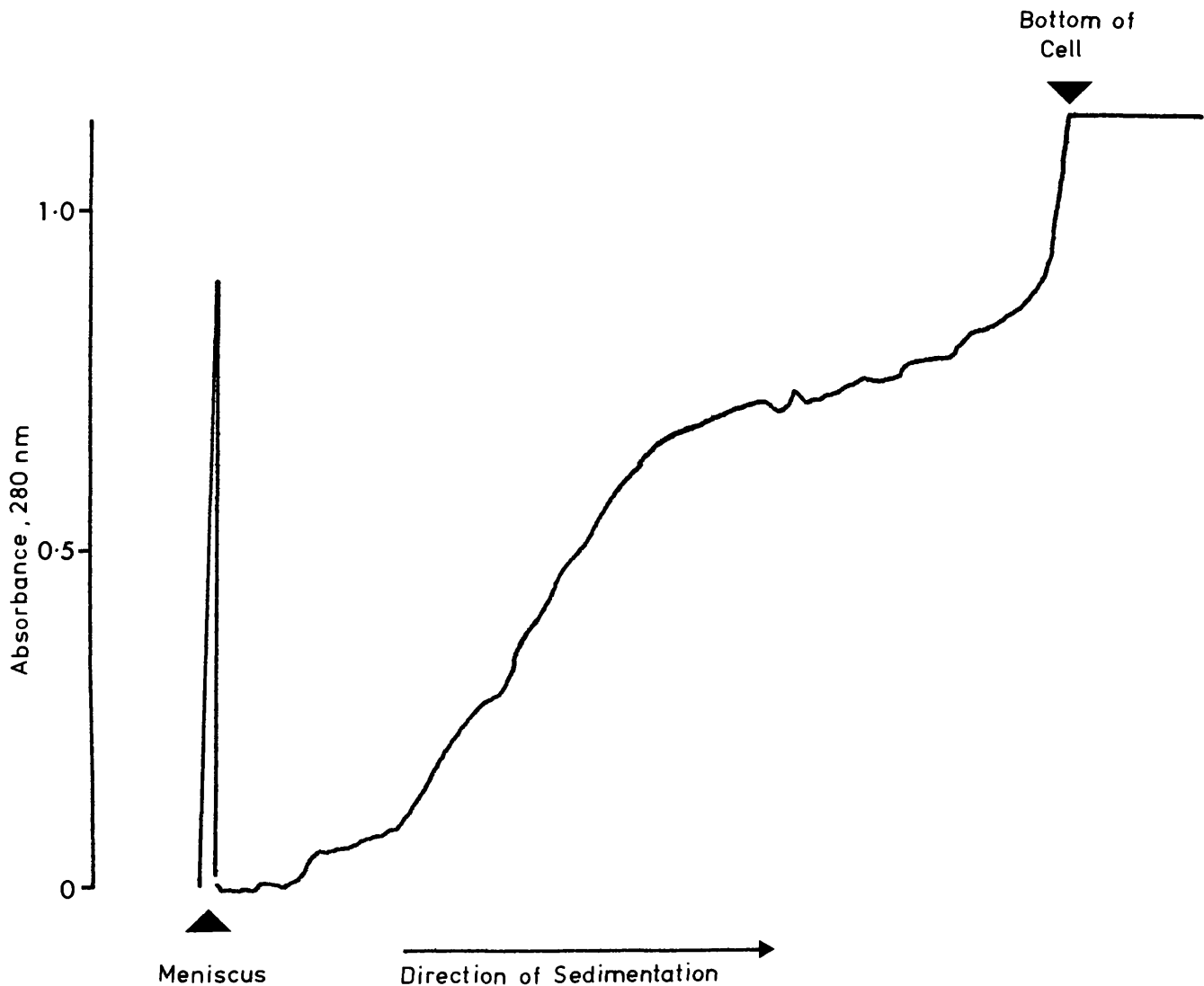
A summary of the purification procedure is presented in table 4. This 7-step process achieved a 600-fold increase in specific activity with a 52% yield. The enzyme was stable for at least

Figure 25. Densitometer Traces of
Citrate Synthase from *B. megaterium*
after SDS-Polyacrylamide Gel
Electrophoresis



(A) Unmodified enzyme; (B) after cross-linking with dimethylsuberimide
and (C) after cross-linking with glutaraldehyde.

Figure 26. Sedimentation-Velocity Analytical Ultracentrifugation of
Purified Citrate Synthase from *B. megaterium*



Centrifugation in 'Tris buffer B' and absorbance scans at 280nm were performed as described in Methods. The scan shown is of a protein solution of concentration 1mg/ml at a rotor speed of 44,388r.p.m.

Table 4. Purification of Citrate Synthase from B. megaterium

Step	Volume (ml)	Total citrate synthase (units)	Total protein (1mg)	Specific activity (units/mg)	Yield (%)
(1) French press extract	110	406	5,800	0.07	-
(2) Supernatant from protamine sulphate treatment	110	474	4,774	0.1	117
(3) Pooled, selected fractions from DEAE-Sephacel ion exchange column	60	431	135	3.2	10.6
(4) Dialysis	60	420	135	3.1	103
(5) Pooled, selected fractions from Matrex Gel Red A -elution with NaCl	11	290	11	26.4	71
(6) Dialysis	11	280	11	25.5	69
(7) Pooled, selected fractions from Matrex Gel Red A -elution with OAA and CoA	4.5	210	4.8	43.8	52

6 months in 'Tris buffer B' at a protein concentration of 1mg/ml.

Determination of the M_r Value of the Native Enzyme

(a) Zonal Gel Filtration and Sedimentation-Velocity Ultracentrifugation

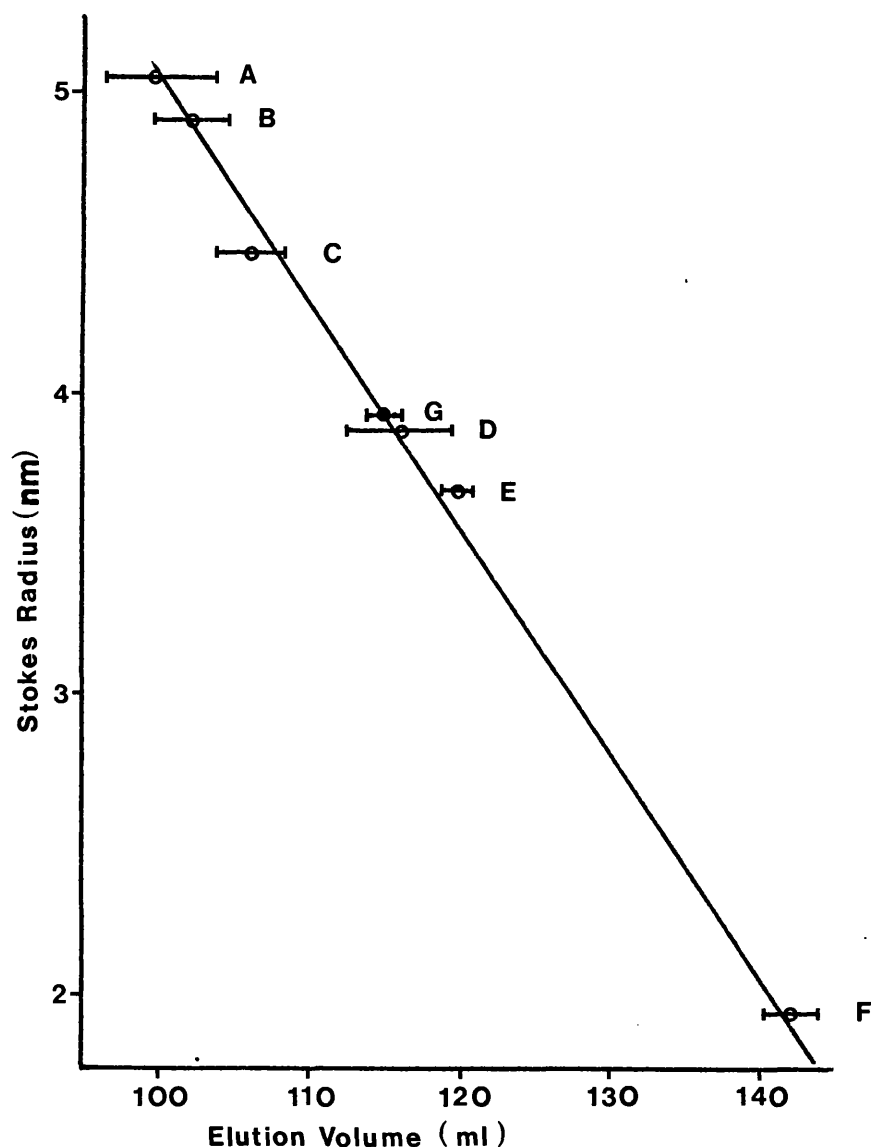
The M_r of B. megaterium citrate synthase was determined by a combination of sedimentation-velocity analysis and zonal gel filtration. The M_r value was then calculated from a combination of the sedimentation coefficient and Stokes radius as described previously.

Gel filtration was carried out in 'Tris buffer B' on a column (70cmx1.6cm) of Sephacryl S-200 (Superfine grade) with the following proteins as standards; beef liver catalase, pig heart fumarase (assayed according to Bergmeyer, 1974c), pig heart lactate dehydrogenase, pig heart malate dehydrogenase, pig heart citrate synthase (run separately from B. megaterium citrate synthase) and whale skeletal-muscle myoglobin. The results are presented in the form of Stokes radius (nm) versus elution volume (ml) in figure 27. B. megaterium citrate synthase was eluted as a single symmetrical peak and its elution volume corresponded to a Stokes radius of 4.0 (± 0.2) nm.

Sedimentation-velocity analysis was performed as described in Methods. The protein sedimented in 'Tris buffer B' as a single species (figure 26). A plot of $\log_n x$ versus time was linear (figure 28a). The values of $s_{20,w}$, determined as described previously, were extrapolated to zero protein concentration (figure 28b) and a value for $s_{20,w}^0$ of 5.2 (± 0.2)S was obtained.

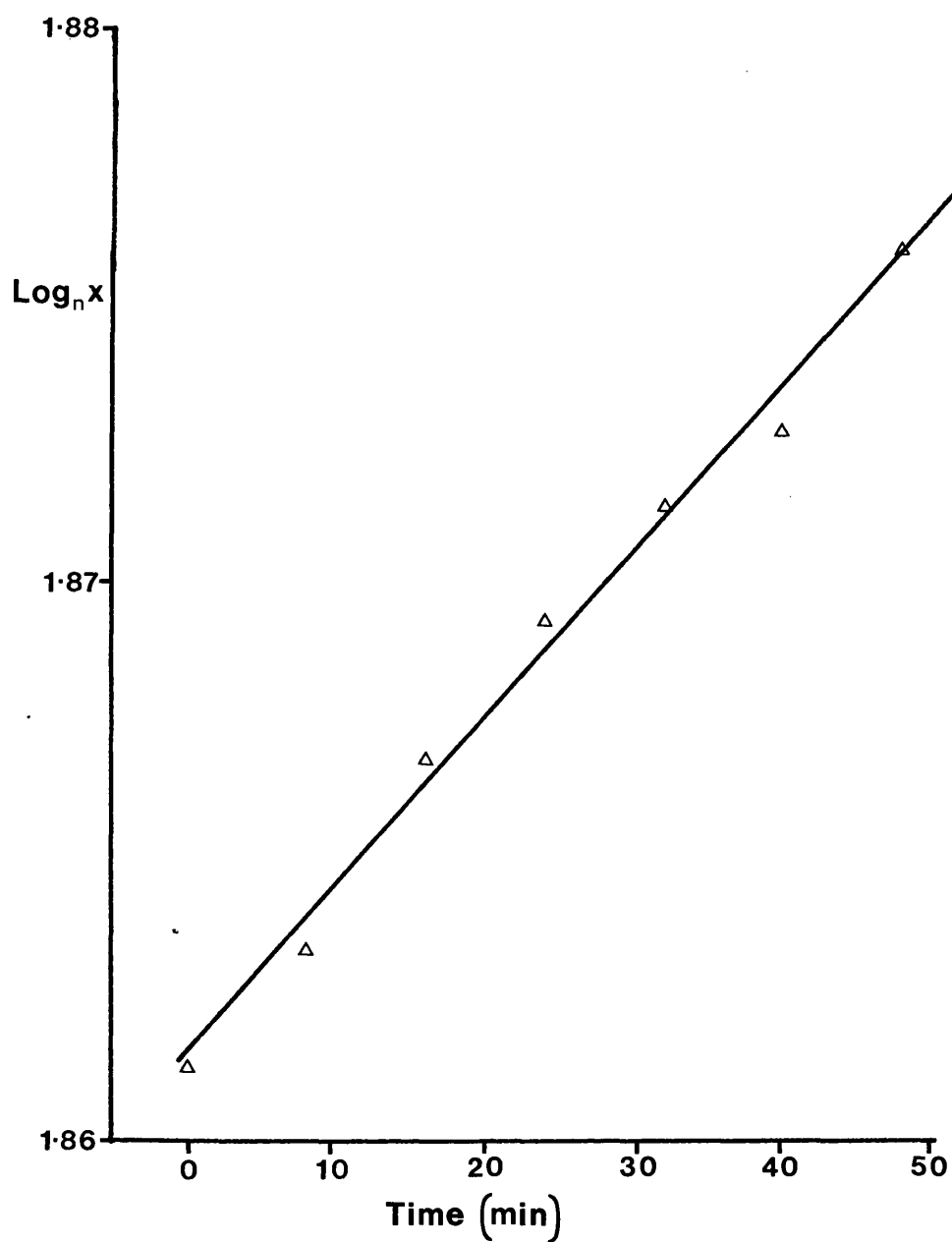
Using a combination of Stokes law and the Svedberg equation, as described previously, and taking \bar{v} as 0.72 from the amino acid composition (table 5), a value of M_r of 84,000 ($\pm 5,000$) was determined.

Figure 27. Gel Filtration on Sephacryl S-200 - Relationship between Stokes Radius (a_0) and Elution Volume



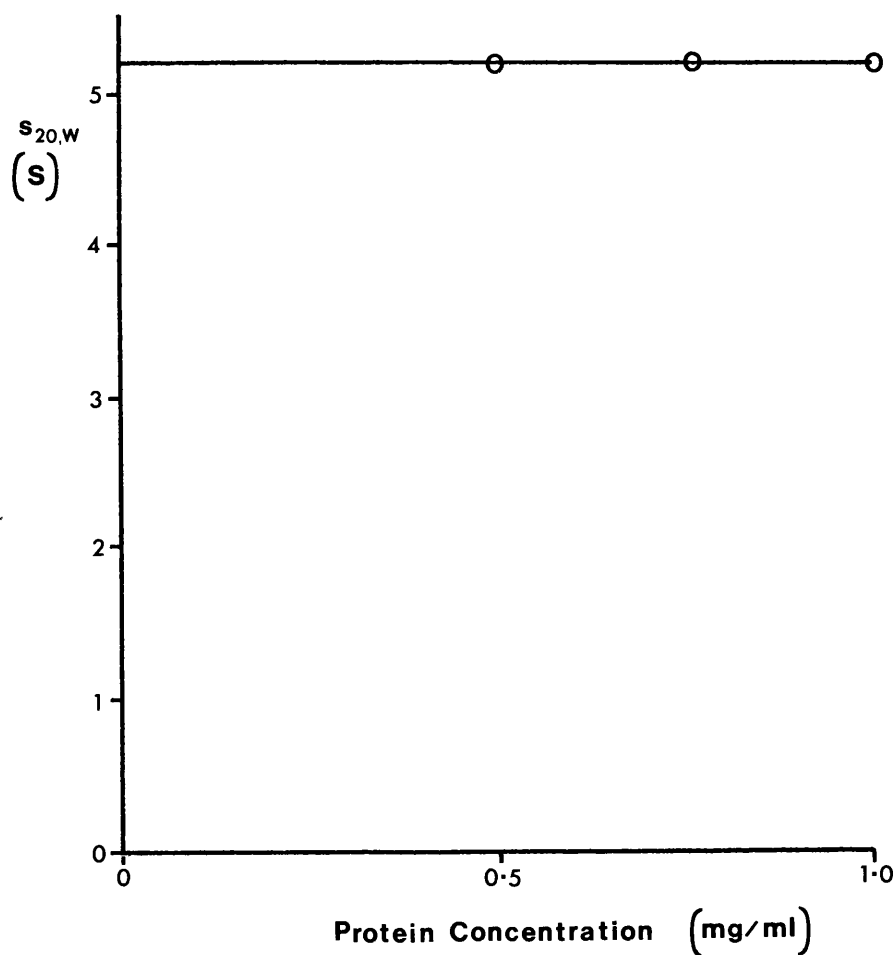
Gel filtration in 'Tris buffer B' was performed as described in Methods. Proteins used as standards were: (A), bovine liver catalase ($a_0=5.05\text{nm}$); (B), pig heart fumarase ($a_0=4.92\text{nm}$); (C), pig heart lactate dehydrogenase ($a_0=4.47\text{nm}$); (D), pig heart citrate synthase ($a_0=3.88\text{nm}$); (E), pig heart malate dehydrogenase ($a_0=3.68\text{nm}$); and (F), whale skeletal-muscle myoglobin ($a_0=1.93\text{nm}$). G represents the citrate synthase from *B. megaterium*. The bars represent the S.E.M. of the volumes at which each protein was eluted in five separate experiments.

Figure 28a Sedimentation-Velocity of Citrate Synthase from *B. megaterium*
Relationship between $\log_n x$ and Time (min)



Sedimentation-velocity analyses were performed in 'Tris buffer B' at 20°C as described in Methods. The figure shown represents a protein concentration of 1mg/ml at a rotor speed of 44388 r.p.m. x is the distance (cm) of the protein boundary from the centre of rotation.

Figure 28b. Sedimentation-velocity Analysis of *B. megaterium*
Citrate Synthase - Determination of $s_{20,w}^0$



B. megaterium citrate synthase was subjected to sedimentation-velocity ultracentrifugation in 'Tris buffer B' as described in the text. The $s_{20,w}^0$ value was calculated by extrapolation to zero protein concentration.

Table 5. Amino Acid Composition of Citrate Synthase from B. megaterium

Amino Acid	Residues/molecule M_r 84,000
Alanine	80.2(± 0.7)
Arginine	38.6(± 0.8)
Aspartate	70.3(± 1.1)
Asparagine	
Cysteine	8.0(± 0.9)
Glutamate	106.9(± 1.5)
Glutamine	
Glycine	76.6(± 2.1)
Histidine	31.9(± 0.9)
Isoleucine	34.0(± 1.6)
Leucine	70.4(± 0.7)
Lysine	38.0(± 0.6)
Methionine	15.1(± 1.0)
Phenylalanine	20.0(± 0.5)
Proline	31.1(± 0.7)
Serine	69.4(± 2.3)
Threonine	46.1(± 3.0)
Tryptophan	1.8(± 0.2)
Tyrosine	21.7(± 1.3)
Valine	40.2(± 0.8)

Values for serine and threonine were extrapolated to zero time and the 72h values only, were used for valine and isoleucine. All other values are means (\pm S.E.M.) from triplicate samples taken after 24h, 48h and 72h hydrolysis.

(b) Sedimentation-Equilibrium Ultracentrifugation

Sedimentation-equilibrium ultracentrifugation was carried out in 'Tris buffer B' at 20°C and 4°C as described in Methods. A plot of $\log_{10} c$ versus r^2 was constructed but was found to be non-linear (figure 29) indicating the presence of more than one molecular species. Therefore a determination of the M_r of B. megaterium citrate synthase using this method was not possible.

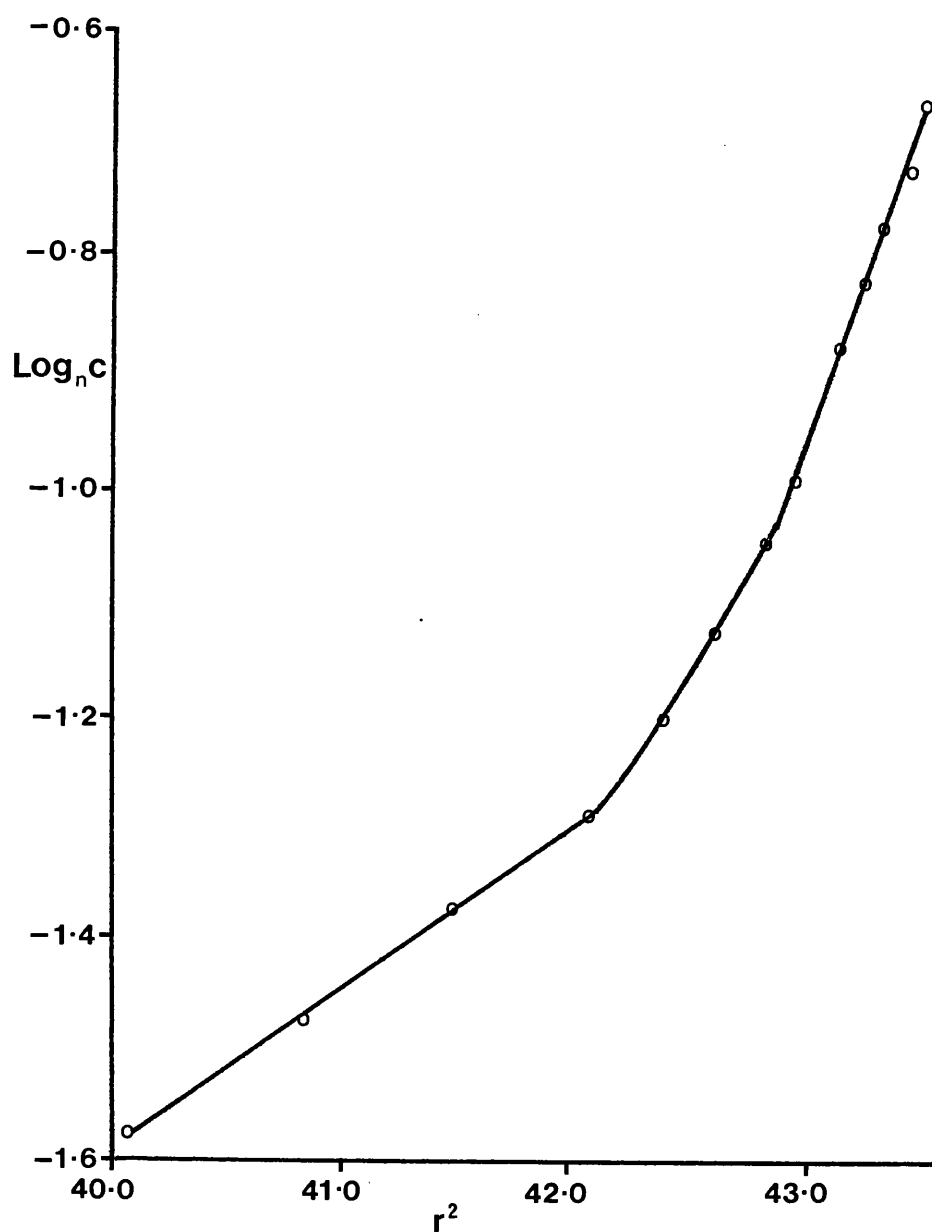
Determination of the M_r Values of the Subunits

(a) SDS-Polyacrylamide Gel Electrophoresis

B. megaterium citrate synthase was analysed by SDS-polyacrylamide gel electrophoresis at pH6.7 as described in Methods. The following proteins were run as standards: E. coli pyruvate decarboxylase, E. coli lipoate acetyltransferase, E. coli dihydrolipoamide dehydrogenase, rabbit muscle fructose biphosphate aldolase, pig heart lactate dehydrogenase and yeast triose phosphate isomerase. The results, plotted as $\log_{10} M_r$ versus Rf, yielded a straight line and are presented in figure 17. B. megaterium citrate synthase ran as a single component and, from the Rf value, a subunit M_r of 43,000 ($\pm 1,000$) was calculated.

The citrate synthase from pig heart, wild-type E. coli and B. megaterium were separated electrophoretically in the presence of SDS (figure 11) despite the similarity in their respective subunit M_r values. This suggests that the order of subunit size is pig heart > E. coli > B. megaterium and the values of M_r are in the range 49,000 - 43,000.

Figure 29. Sedimentation-Equilibrium Analysis of Purified Citrate Synthase from *B. megaterium* - Relationship between $\log_n c$ and r^2



Protein concentration was 0.5mg/ml in 'Tris buffer B'. Centrifugation was performed at 4°C at a rotor speed of 12520 r.p.m. c is the protein concentration (mg/ml) at r , the distance (cm) from the centre of rotation.

(b) Gel Filtration in 6M Guanidinium Chloride

B. megaterium citrate synthase was also analysed by gel filtration under denaturing conditions in 6M guanidinium chloride using a column (76cmx1.6cm) of Sephacryl S-300 (superfine grade). The enzyme was radiolabelled as described in Methods. The following radiolabelled proteins were also run as standards: E. coli β -galactosidase, rabbit muscle phosphorylase a, bovine serum albumin, rabbit muscle fructose biphosphate aldolase, rabbit muscle glyceraldehyde 3-phosphate dehydrogenase and horse heart cytochrome c. The results were plotted in the form $\log_{10} 100K_d$ versus $N^{2/3}$ (Rydén, 1971) and are presented in figure 18. K_d is the distribution coefficient of the polypeptide and N the number of amino acid residues in the polypeptide chain. A linear relationship existed between $\log_{10} 100K_d$ and $N^{2/3}$. B. megaterium citrate synthase behaved as a single component and the K_d determined corresponded to a value of N of 368 (± 44) amino acids per polypeptide chain.

From the amino acid analysis of B. megaterium citrate synthase (table 5) and the value determined for N , a value for the subunit M_r was calculated as 38,600 ($\pm 4,600$). This is in good agreement with the value obtained by SDS-polyacrylamide gel electrophoresis.

Determination of the Number of Subunits

The results of the determination of the M_r values of the native enzyme and subunit protein, 84,000 and 41,000 respectively, suggest that citrate synthase from B. megaterium may be dimeric. The polymeric nature of the enzyme may be investigated using cross-linking reagents as described previously for E. coli citrate synthase.

Pure B. megaterium citrate synthase was cross-linked using either dimethylsuberimide or glutaraldehyde as described in Methods. The cross-linked protein was then analysed by SDS-polyacrylamide gel electrophoresis. After staining, the gels were scanned and the results are shown in figure 25. When either dimethylsuberimide or glutaraldehyde were used, two bands were detected on the gels indicating B. megaterium citrate synthase is a dimer. The ratio of the M_r values of the two molecular species was determined to be approximately 2:1 from their R_f values.

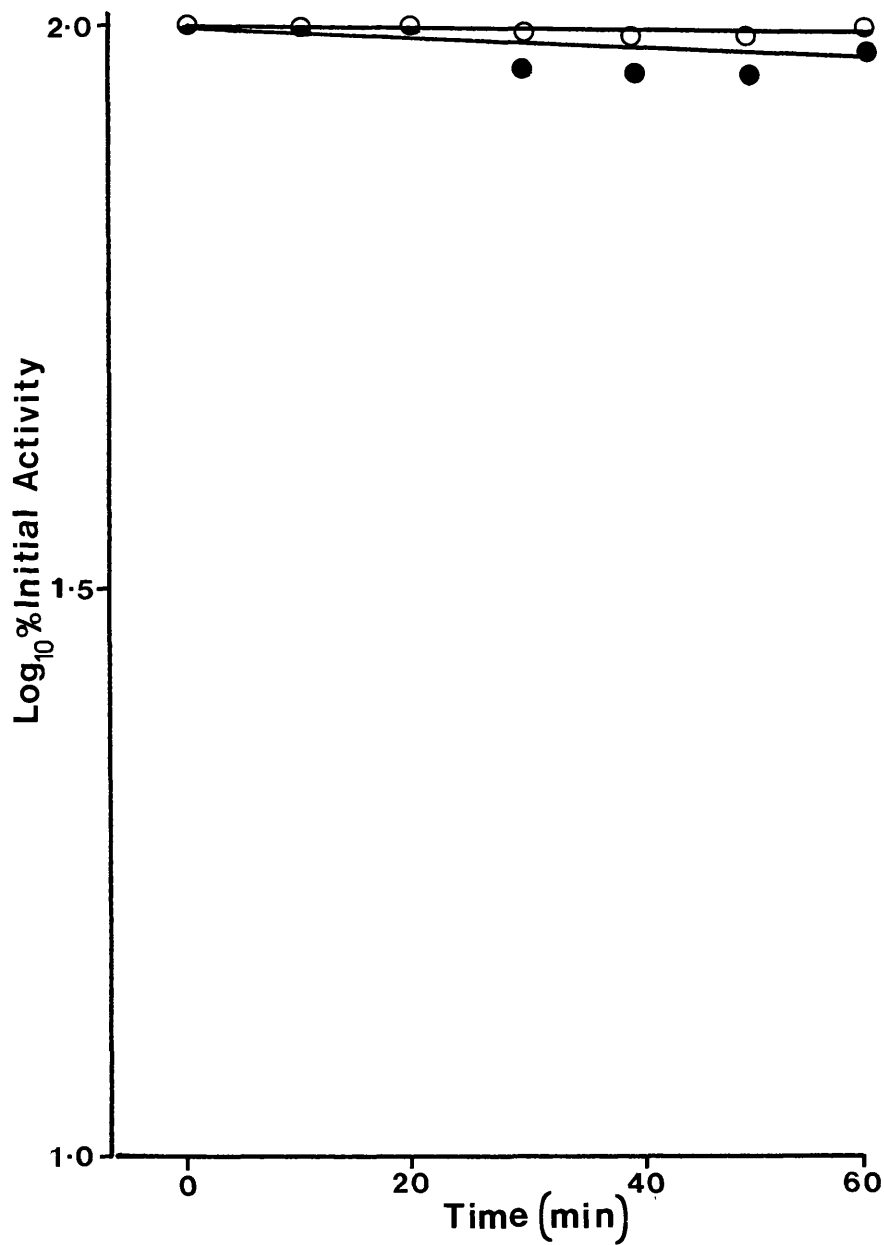
Modification of B. megaterium Citrate Synthase with Specific Chemical Reagents

(a) DTNB

DTNB is a reagent which specifically attacks thiol groups. B. megaterium citrate synthase (0.1mg) was incubated in the dark at 20°C in 1ml of 'Tris buffer B' containing 0.1mM DTNB. Samples were removed at intervals for assay of citrate synthase activity in both the absence and presence of 5mM ATP. A time course of citrate synthase activity and inhibition by 5mM ATP was constructed and the results are shown in figure 30. There was no significant inactivation of B. megaterium citrate synthase under these conditions, nor was there any desensitization to ATP inhibition.

The absorbance at 412nm of the inactivation mixture remained zero throughout, indicating no thiol groups were modified. The experiment was repeated using 1mM DTNB and once again the enzyme proved insensitive.

Figure 30. Inactivation of *B. megaterium* Citrate Synthase and Desensitization to ATP by DTNB.



The citrate synthase from *B. megaterium* was incubated with 0.1mM DTNB at pH8.0 at 20°C as described in the text. The results are expressed as log₁₀% initial activity versus time: (O), enzyme activity; (●) inhibition by ATP.

(b) Diethylpyrocarbonate (DEPC)

Diethylpyrocarbonate (DEPC) is a specific reagent for the modification of histidine residues (Ovádi et al., 1967) at approximately pH6.0.

The concentration of DEPC in commercial preparations was determined from the increase in absorbance at 240nm after reaction with excess (0.3mM) imidazole at pH8.0. The molar absorbance of the product was taken to be 3,200 l/mol/cm (Ovádi et al., 1967).

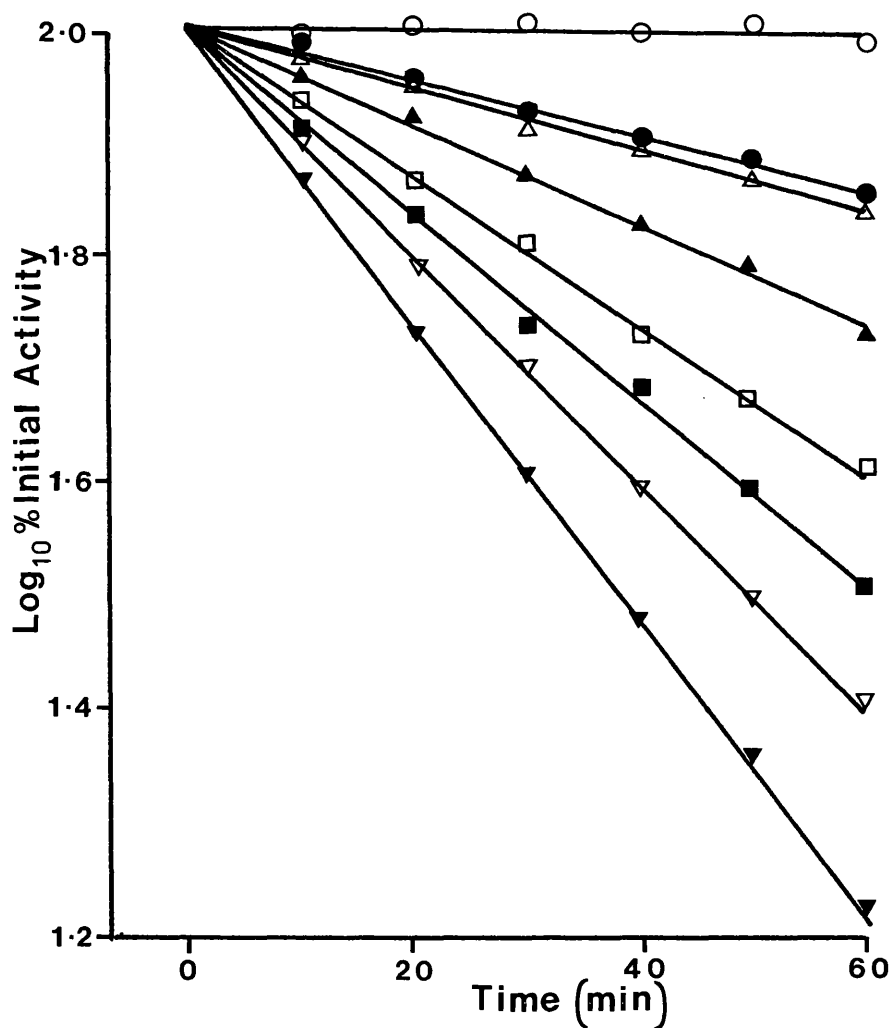
(i) Protection by Substrates and ATP against Inactivation

Modification of B. megaterium citrate synthase (50µg) with DEPC was carried out at 4°C in 1ml of 50mM acetate buffer, containing 20% (v/v) glycerol. DEPC was added at various concentrations as a freshly prepared aqueous solution. Samples were removed periodically for assay of citrate synthase activity.

Treatment of B. megaterium citrate synthase with a range of concentrations of DEPC resulted in inactivation of the enzyme and produced linear, monophasic semi-log plots (figure 31). When the observed rate constants of inactivation (k_{obs}) were plotted against the concentration of DEPC a straight line was produced (figure 32) indicating the rate of inactivation was directly proportional to reagent concentration.

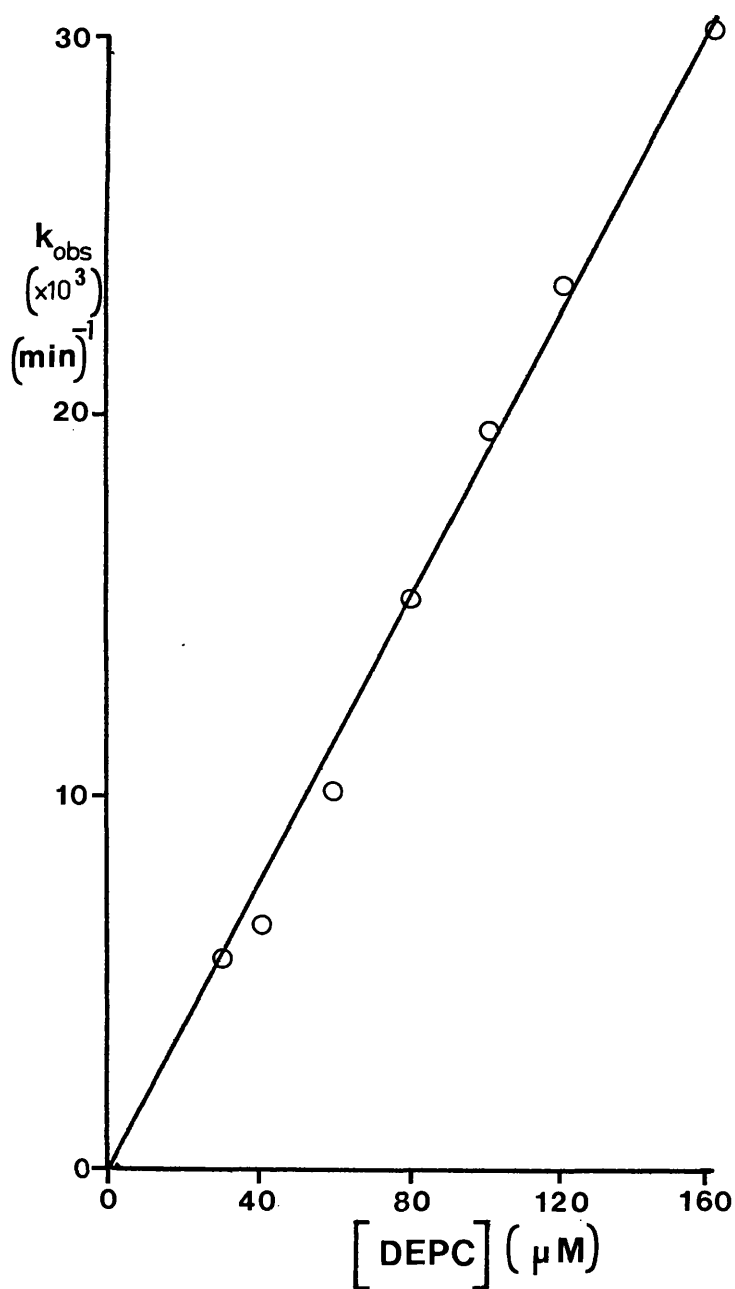
Inactivation was carried out using 160 µM DEPC with enzyme alone and in the presence of enzyme-saturating concentrations of acetyl-CoA (5mM \pm 100Km), oxaloacetate (1mM \pm 100Km) and ATP (5mM \pm 5Ki). The results, presented as semi-log plots, are shown in figure 33. In the absence of either substrate or ATP the enzyme

Figure 31. Inactivation of Citrate Synthase from *B. megaterium*
by a Range of Concentrations of DEPC, Expressed as
 \log_{10} % Initial Activity versus Time



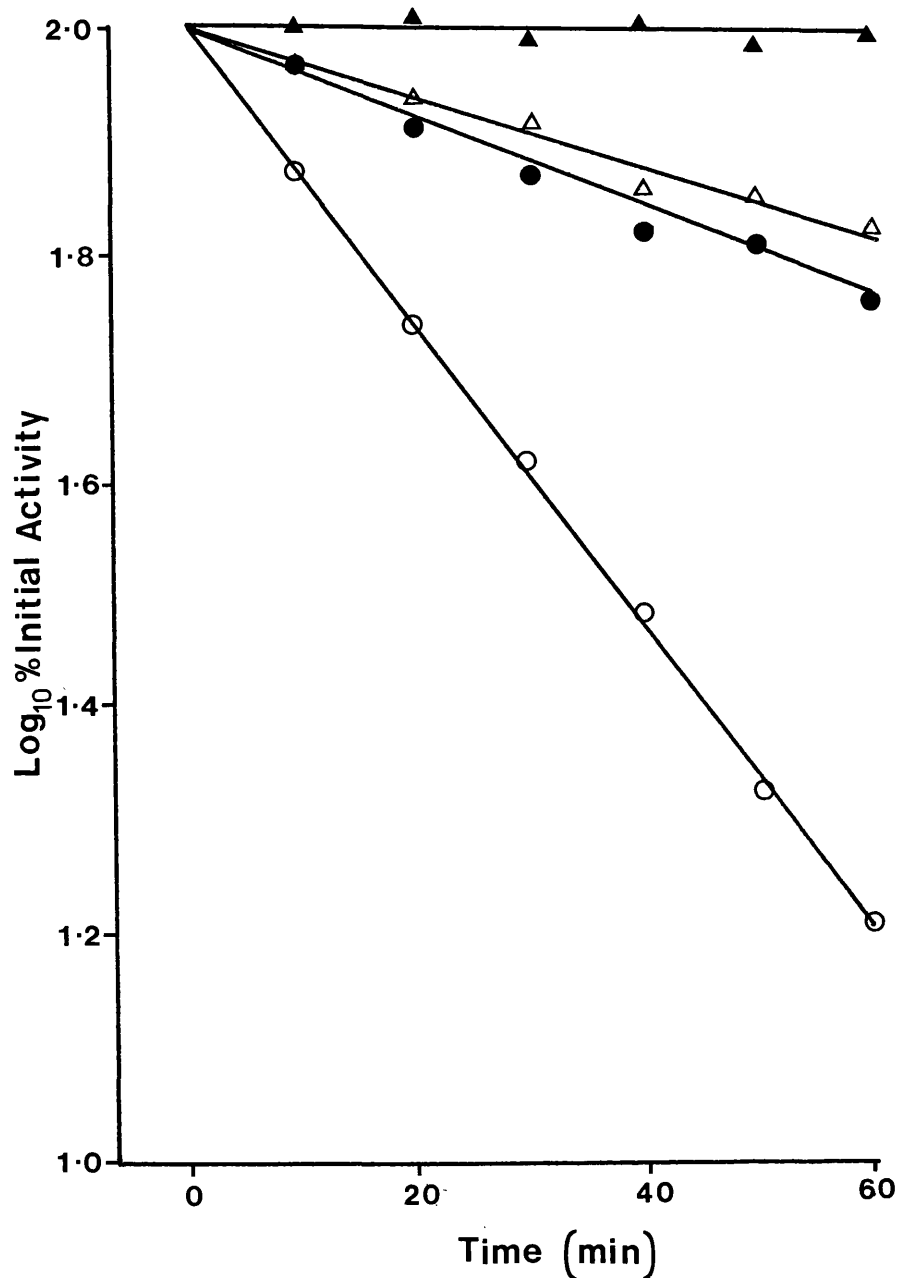
Purified *B. megaterium* citrate synthase (50ug/ml) was incubated at pH6.5 at 4°C with a range of concentrations of DEPC as described in Methods. Concentrations of DEPC used were: (O) 0 μ M; (●), 30 μ M; (Δ), 40 μ M; (\blacktriangle), 60 μ M; (\square), 80 μ M; (\blacksquare), 100 μ M; (∇), 120 μ M; and (\blacktriangledown), 160 μ M.

Figure 32. Inactivation of Citrate Synthase from *B. megaterium* by DEPC - Relationship between the Observed Rate Constant of Inactivation (k_{obs}) and DEPC Concentration



The observed rate constant (k_{obs}) of inactivation at each concentration of DEPC was determined from figure 31.

Figure 33. Inactivation of Citrate Synthase from *B. megaterium* by DEPC
in the Presence of Enzyme-Saturating Concentrations of
Oxaloacetate, Acetyl-CoA and ATP

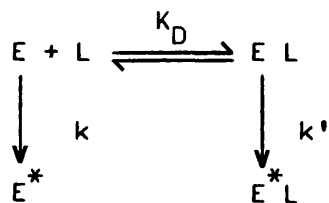


B. megaterium citrate synthase was incubated with 160 μ M DEPC at pH6.5 at 4°C in the presence of: (○), no addition; (●), 5mM acetyl-CoA; (△), 5mM ATP; and (▲), 1mM oxaloacetate. The results are expressed as log₁₀% initial activity versus time.

was inactivated with a pseudo first-order rate constant of $3.0 (\pm 0.2) \times 10^{-2} \text{ min}^{-1}$. This corresponds to a true second-order rate constant of $187.5 (\pm 12.5) \text{ M}^{-1} \text{ min}^{-1}$. Oxaloacetate (1mM) afforded complete protection whereas both acetyl-CoA (5mM) and ATP (5mM) decreased the inactivation pseudo first-order rate constant to $0.7 (\pm 0.1) \times 10^{-2} \text{ min}^{-1}$.

(ii) Determination of the Dissociation Constant (K_D) for Oxaloacetate, Acetyl-CoA and ATP using Inactivation of Citrate Synthase by DEPC.

Protection of an enzyme by ligands against chemical modification can be described by the following relationship:



where E is unmodified, active enzyme; E^* is the modified enzyme; L is the ligand; K_D is the dissociation constant of the enzyme-ligand complex; k is the rate constant for the modification of free enzyme; and k' is the rate constant for the modification of ligand-bound enzyme. The following equation can be derived to describe the inactivation of an enzyme by a chemical reagent (see appendix):

$$\frac{1}{k_{\text{obs}} - k} = \frac{K_D}{[L] (k' - k)} + \frac{1}{(k' - k)}$$

where k_{obs} is the observed rate constant of inactivation. Thus by carrying out incubations of the enzyme with the chemical reagent in the presence of a range of concentrations of ligand [L], a

plot of: $\frac{1}{k_{\text{obs}} - k}$ versus $\frac{1}{[L]}$

will produce a vertical intercept of: $\frac{1}{k' - k}$

and a slope of: $\frac{K_D}{k' - k}$

Thus values for k' and K_D can be determined. The value of k' , compared with that of k , may be considered a measure of the effectiveness of protection offered by a ligand - the lower k' , the greater the degree of protection. In the case where k' is zero, the ligand affords complete protection against inactivation.

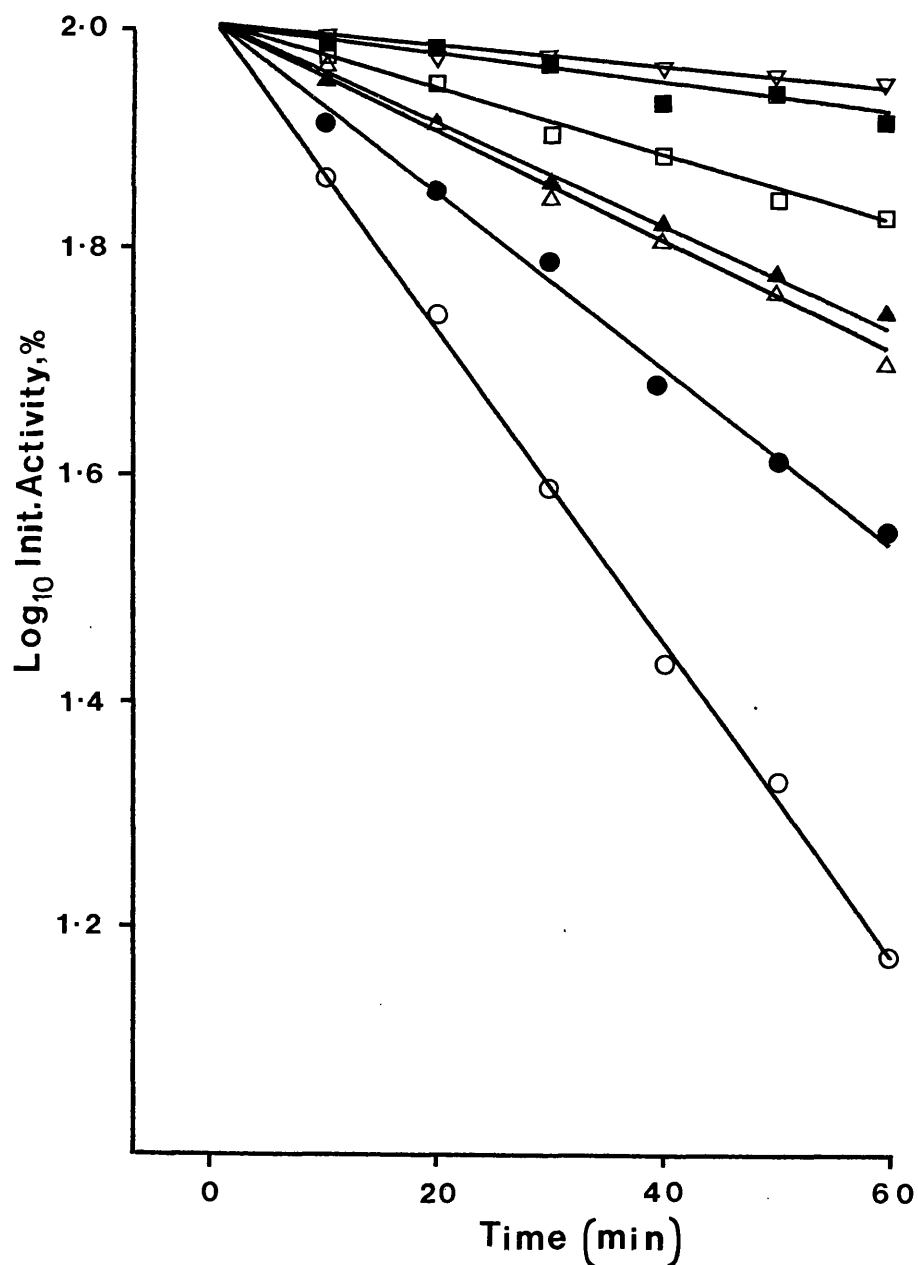
Applying this general example to B. megaterium citrate synthase, modification by DEPC causes inactivation which can be prevented by the ligands, oxaloacetate, acetyl-CoA and ATP.

B. megaterium citrate synthase was incubated with DEPC (160 μ M), using a range of concentrations of either oxaloacetate, acetyl-CoA or ATP. The results are presented in figures 34, 35 and 36. From the slopes of these semi-log plots, values for k_{obs} were determined and secondary plots of $\frac{1}{k_{\text{obs}} - k}$ versus $\frac{1}{[L]}$

were constructed (figures 37, 38 and 39). The dissociation constants (K_D) were determined for oxaloacetate, acetyl-CoA and ATP as described above. These were 2.3 (± 0.2) μ M for oxaloacetate, 51.0 (± 4.9) μ M for acetyl-CoA and 27.7 (± 4.5) μ M for ATP.

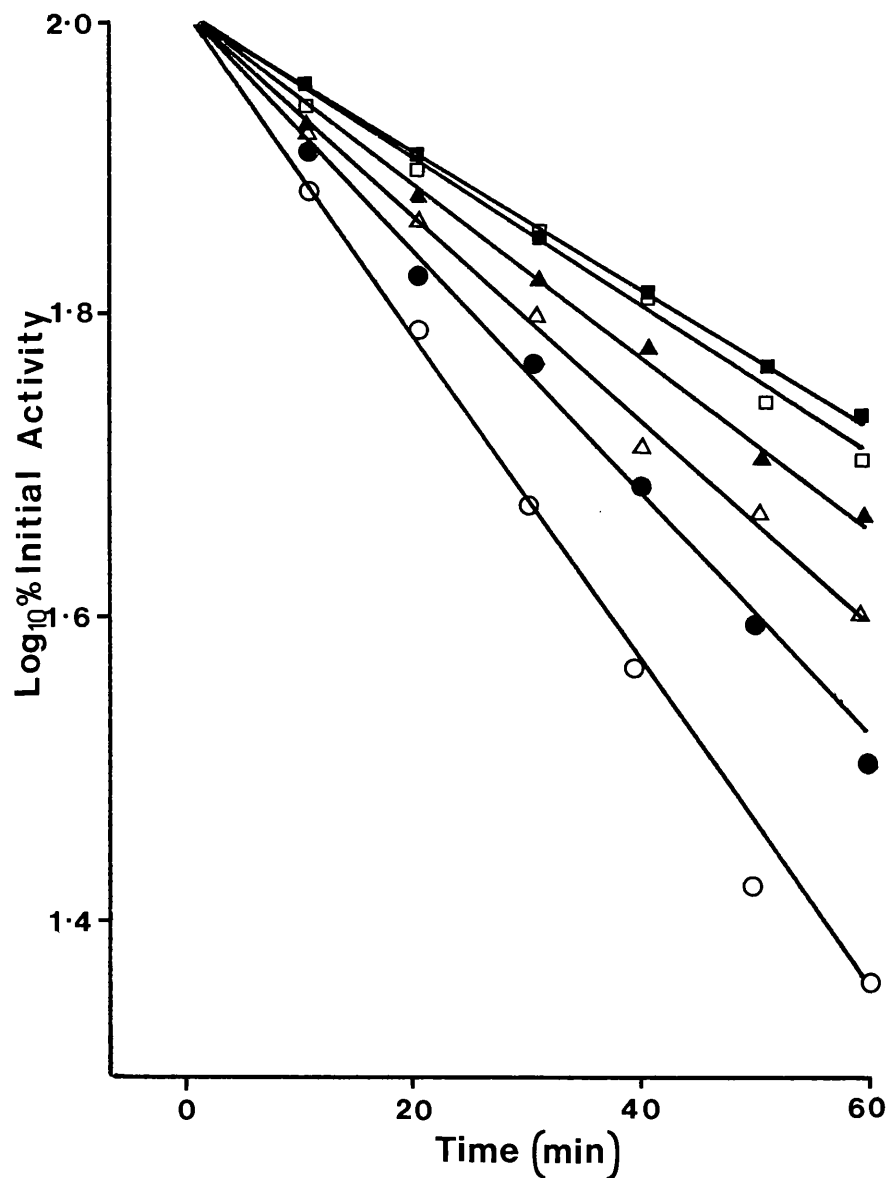
The values obtained for k' were: 0.6 (± 0.1) $\times 10^{-2} \text{ min}^{-1}$ using acetyl-CoA; 0.4 (± 0.4) $\times 10^{-2} \text{ min}^{-1}$ using ATP;

Figure 34. Inactivation of Citrate Synthase from *B. megaterium* by DEPC in the Presence of a Range of Concentrations of Oxaloacetate



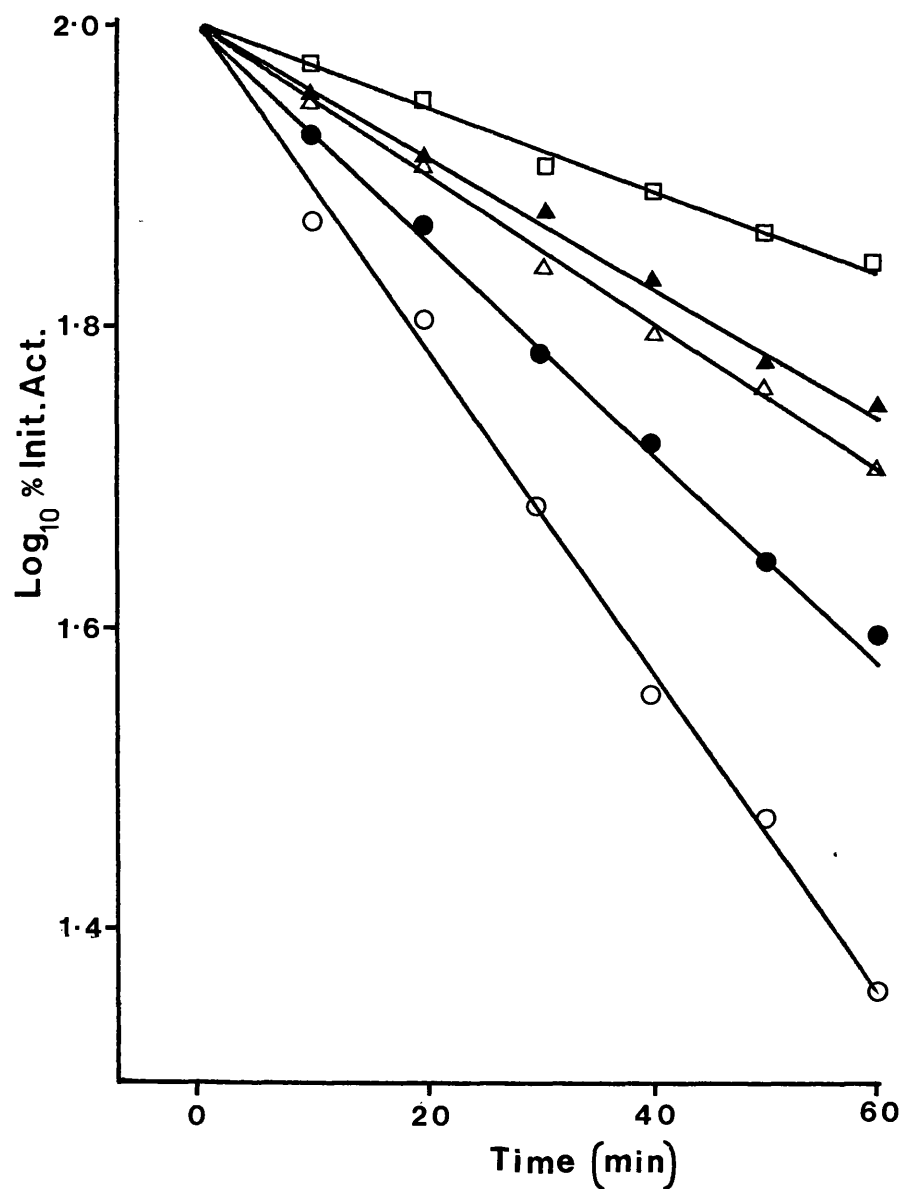
B. megaterium citrate synthase was incubated with 160 μ M DEPC in the presence of various concentrations of oxaloacetate as described in the text. Concentrations of oxaloacetate used were: (o), 0 μ M; (\bullet), 2 μ M; (Δ), 4 μ M; (\blacktriangle), 6 μ M; (\square), 8 μ M; (\blacksquare), 20 μ M; and (∇), 40 μ M. The results are expressed as \log_{10} % initial activity versus time.

Figure 35. Inactivation of Citrate Synthase from *B. megaterium* by DEPC in the Presence of a Range of Concentrations of Acetyl-CoA



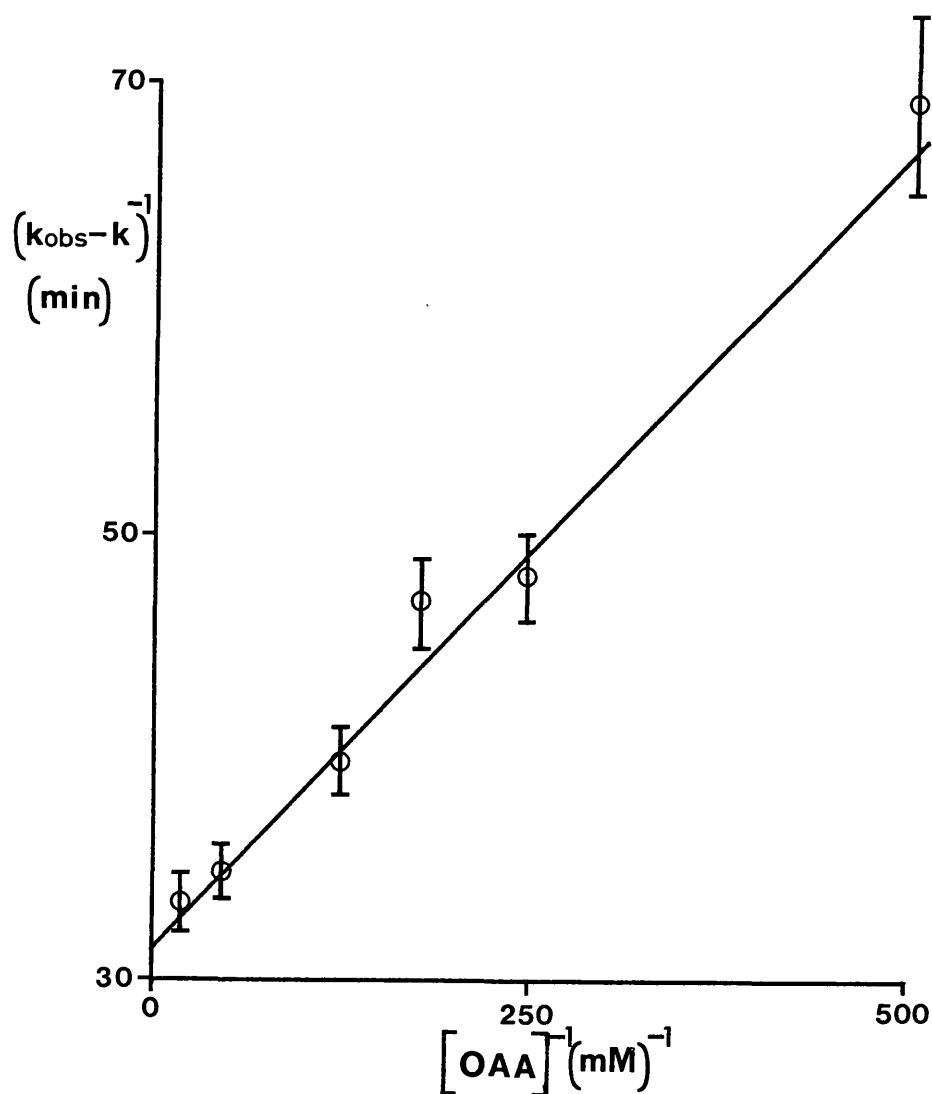
B. megaterium citrate synthase was incubated with 160 μM DEPC in the presence of various concentrations of acetyl-CoA as described in the text. Concentrations of acetyl-CoA used were: (○), 0 μM; (●), 25 μM; (△), 50 μM; (▲), 100 μM; (◻), 150 μM; and (■), 200 μM. The results are expressed as log₁₀% initial activity versus time.

Figure 36. Inactivation of Citrate Synthase from *B. megaterium* by DEPC in the Presence of a Range of Concentrations of ATP



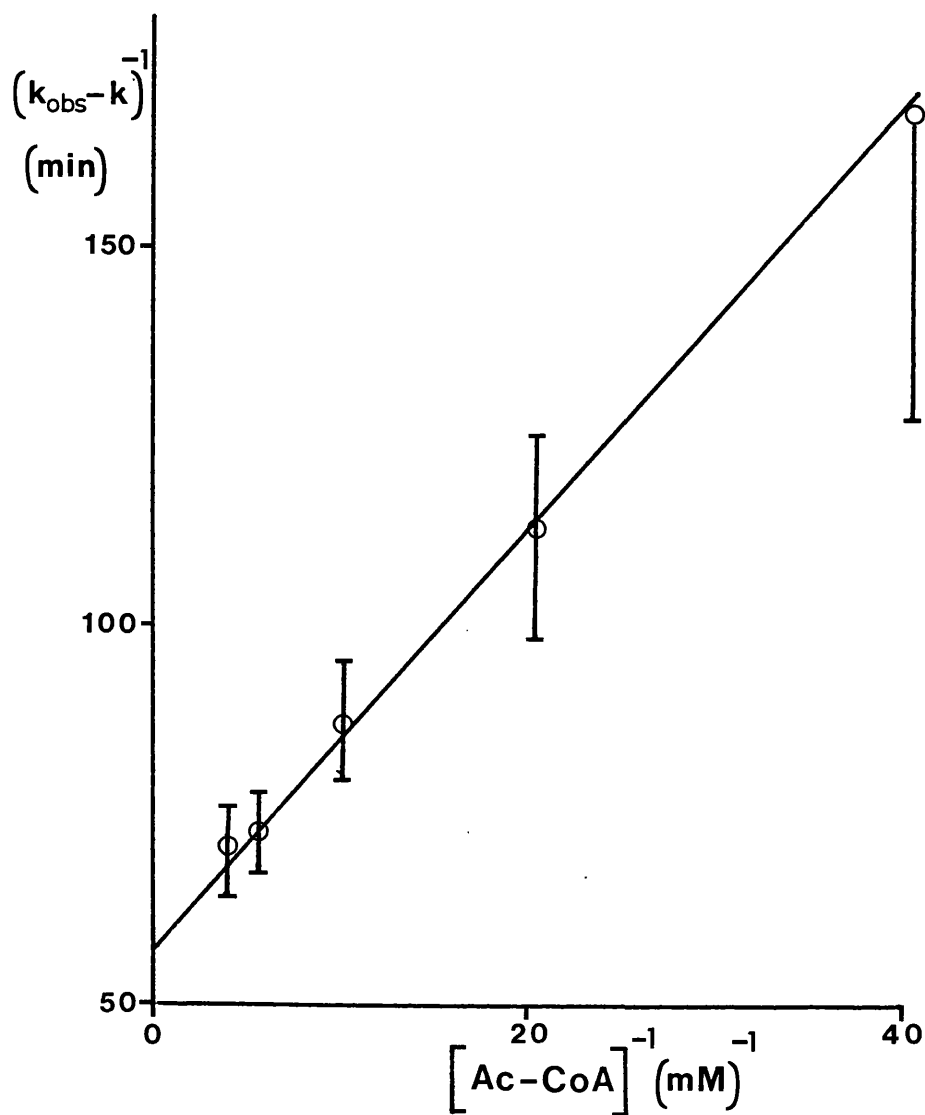
B. megaterium citrate synthase was incubated with 160 μ M DEPC in the presence of various concentrations of ATP as described in the text. Concentrations of ATP used were: (○), 0 μ M; (●), 20 μ M; (△), 50 μ M; (▲), 80 μ M; and (□), 150 μ M. The results are expressed as \log_{10} % initial activity versus time.

Figure 37. Secondary Plot of $(k_{\text{obs}} - k)^{-1}$ versus $[\text{oxaloacetate}]^{-1}$ for the Determination of the Value of K_D of Oxaloacetate for *B. megaterium* Citrate Synthase



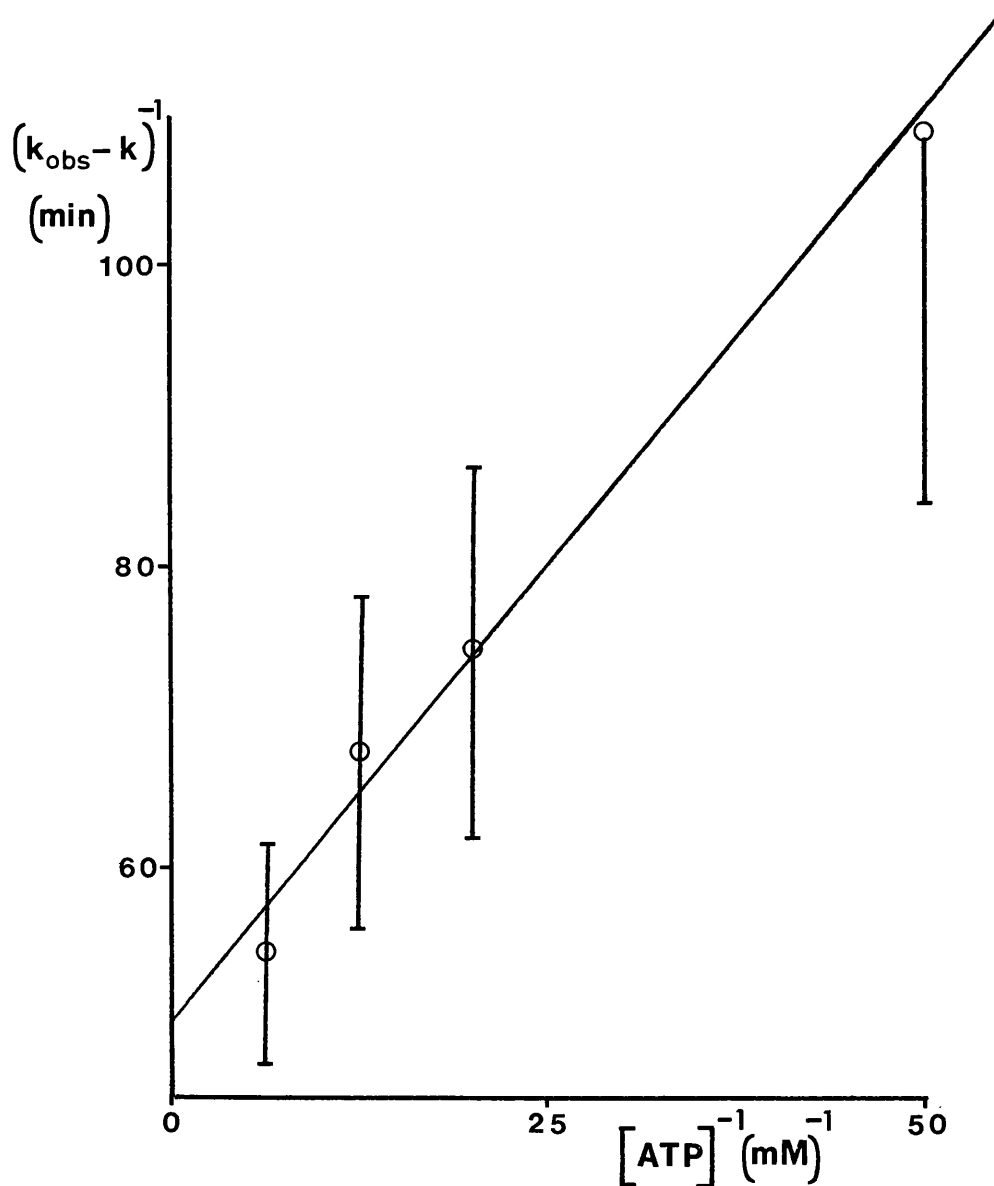
Values for the rate constants k_{obs} and k , and the S.E.M. values for $(k_{\text{obs}} - k)^{-1}$ (represented by bars) were determined from figure 34. k_{obs} and k are defined in the text.

Figure 38. Secondary Plot of $(k_{\text{obs}} - k)$ versus $[\text{acetyl-CoA}]^{-1}$ for the Determination of the Value of K_D of Acetyl-CoA for B. megaterium Citrate Synthase



Values for the rate constants k_{obs} and k and the S.E.M. values for $(k_{\text{obs}} - k)$ (represented by bars) were determined from figure 35. k_{obs} and k are defined in the text.

Figure 39. Secondary Plot of $(k_{\text{obs}} - k)^{-1}$ versus $[\text{ATP}]^{-1}$ for the Determination of the Value of K_D of ATP for B. megaterium Citrate Synthase



Values for the rate constants k_{obs} and k and the S.E.M. values for $(k_{\text{obs}} - k)$ (represented by bars) were determined from figure 36. k_{obs} and k are defined in the text.

and $0.0 (\pm 0.2) \times 10^{-2} \text{ min}^{-1}$ using oxaloacetate. These results suggest that only oxaloacetate is capable of completely protecting the enzyme against modification by DEPC. The values of k' are not significantly different from the values of k_{obs} when using enzyme saturated with ligands (table 6). This is to be expected, as in the presence of saturating concentrations of ligand, most of the enzyme will be in the form EL and the observed rate constant of inactivation (k_{obs}) will therefore be equal to k' .

(iii) Determination of the Number of Modified Histidine Residues per Enzyme Molecule

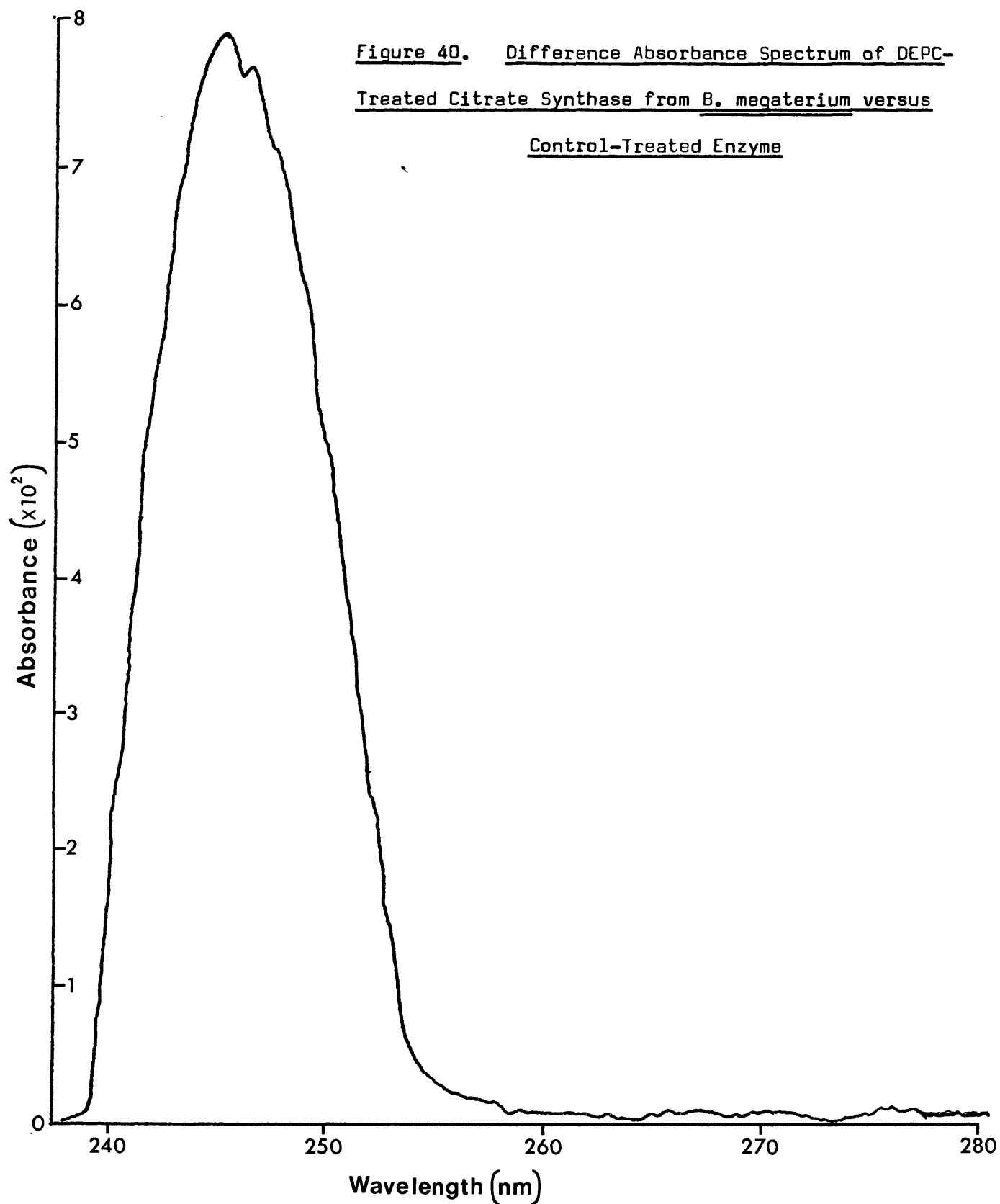
A method exists for determining the number of histidine residues per citrate synthase molecule modified by DEPC. This relies on the spectroscopic determination of ethoxyformyl histidine which has a characteristic absorbance peak at approximately 240nm (Ovádi et al., 1967).

B. megaterium citrate synthase (1.2mg/ml) was incubated overnight (16h) at 4°C with $160\mu\text{M}$ DEPC in 50mM sodium acetate buffer, pH6.5, containing 20% (v/v) glycerol. A control sample was prepared with no DEPC present. The DEPC-treated citrate synthase was found to have lost 87% of its original enzyme activity, whereas the control enzyme had not lost any significant activity over the same period. A difference spectrum of DEPC-treated enzyme versus control enzyme was run from a wavelength of 220nm to 280nm using a Pye Unicam SP8-100 dual-beam spectrophotometer. The spectrum is shown in figure 40 and reveals an absorbance peak at 246nm, characteristic of ethoxyformyl histidine (Ovádi et al., 1967; Mùhlrad et al., 1967; Elödi, 1968). From the change in absorbance at 246nm (0.079)

Table 6. Comparison of the Pseudo First-Order Rate Constants of
Inactivation of Citrate Synthase from *B. megaterium*
by DEPC

Ligand	k_{obs} (min^{-1})	k' (min^{-1})
Oxaloacetate	$0 (\pm 1) \times 10^{-3}$	$0 (\pm 2) \times 10^{-3}$
Acetyl-CoA	$7 (\pm 1) \times 10^{-3}$	$6 (\pm 1) \times 10^{-3}$
ATP	$7 (\pm 1) \times 10^{-3}$	$4 (\pm 4) \times 10^{-3}$

k_{obs} was determined in the presence of enzyme-saturating concentrations of ligand. k' was determined as described in the text from figures 37, 38 and 39.



Citrate synthase (1.2mg/ml) was treated overnight at 4°C with 160 μ M DEPC as described in the text.

and taking the molar absorbance of ethoxyformyl histidine to be 3,200 l/mol/cm (Ovádi et al., 1967) and the M_r of B. megaterium citrate synthase to be 84,000, the number of histidines modified per enzyme molecule was calculated to be 1.7. This corresponded to a 87% loss in enzyme activity and thus a 100% loss in activity would necessitate the modification of 1.9 histidine residues. If the B. megaterium citrate synthase subunits are identical, it may be suggested that one histidine residue is modified per subunit and that this histidine is essential for catalytic activity. The difference spectrum also revealed no change in absorbance at 280nm suggesting that in this case, both tryptophan and tyrosine residues were not modified by DEPC.

(iv) Treatment of DEPC-Modified Citrate Synthase with Hydroxylamine

Hydroxylamine at pH7.0 is known to remove the ethoxyformyl group from histidine (Melchior and Fahrney, 1970) thus allowing regeneration of native, active enzyme. Hydroxylamine treatment was carried out as follows. The DEPC-inactivated citrate synthase was dialysed exhaustively against 20mM sodium phosphate buffer, pH7.0, containing 20% (v/v) glycerol and then incubated in this buffer containing 0.25M neutralized hydroxylamine. After 30min at room temperature the enzyme was dialysed exhaustively against 'Tris buffer B' and then assayed. It was found that no activity had been regained.

When a difference spectrum of this hydroxylamine-treated enzyme was run, it was found that the absorbance peak at 246nm had completely disappeared suggesting regeneration of the histidine residues. When native active B. megaterium citrate synthase was treated with hydroxylamine in the same manner as above, activity was completely lost, although native enzyme incubated in 20mM phosphate buffer, pH7.0, alone, did not lose any significant activity.

These results indicate that hydroxylamine itself inactivates the enzyme. Under certain conditions, pH 10.5 and 35°C, hydroxylamine cleaves polypeptide chains at asparagine-glycine bonds (Bornstein, 1970). To discover whether B. megaterium citrate synthase was undergoing cleavage at pH 7.0, hydroxylamine-treated enzyme was analysed by SDS-polyacrylamide gel electrophoresis and the results shown in figure 41. There was no significant difference between the mobilities of untreated and hydroxylamine-treated enzyme. The means of inactivation in the presence of hydroxylamine remains obscure.

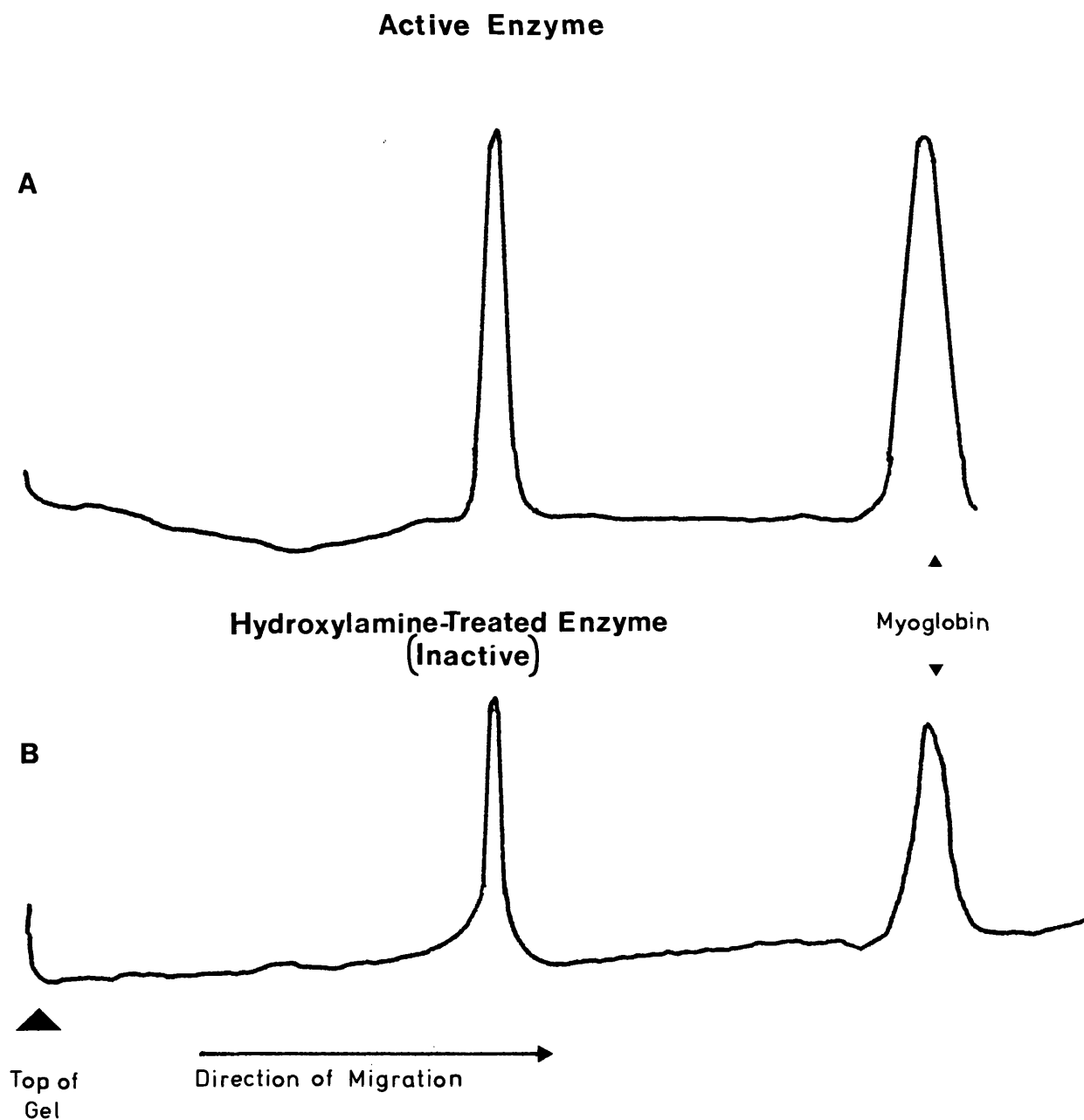
Kinetic Studies and Regulation

(a) At pH 8.0

Plots of (v) versus $[S]$ for each substrate were hyperbolic when the enzyme was assayed in 'Tris buffer B' (figure 42). The K_m^{app} for each substrate was determined at various fixed concentrations of the other. The values of K_m^{app} were determined using direct linear plots (Eisenthal and Cornish-Bowden, 1974) and the data are presented in the form: $\frac{[S]}{v}$ versus $[S]$ (figure 43).

The lines were constructed from the values of K_m^{app} and V_{max}^{app} obtained by the direct linear plots. The K_m^{app} values determined for oxaloacetate were independent of the acetyl-CoA concentrations used (figure 44a). Similarly the K_m^{app} values determined for acetyl-CoA were independent of the concentration of oxaloacetate used (figure 44b). By plotting values of V_{max}^{app} versus $[S]$ on the direct linear plots, the value for the K_m of one substrate at infinite concentration of the other could be obtained. These results are presented in the form:

Figure 41. Densitometer Traces of Purified Citrate Synthase from
B. megaterium after SDS-Polyacrylamide Gel Electrophoresis

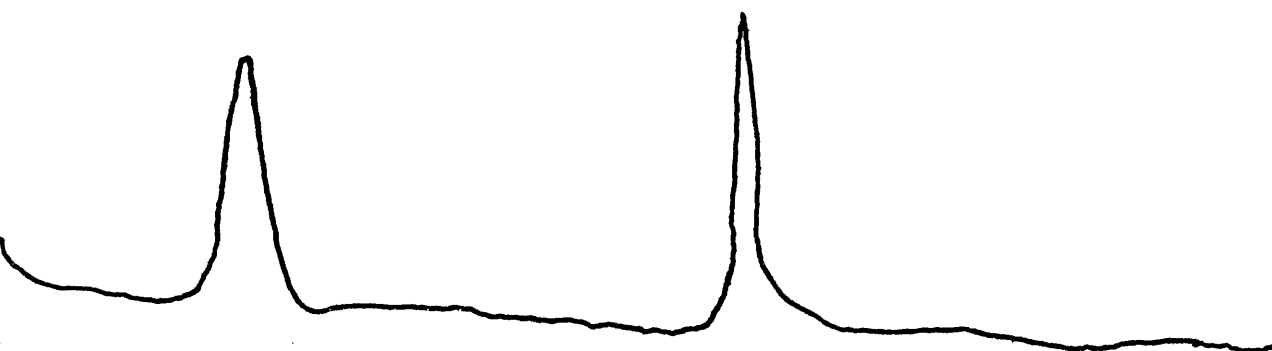
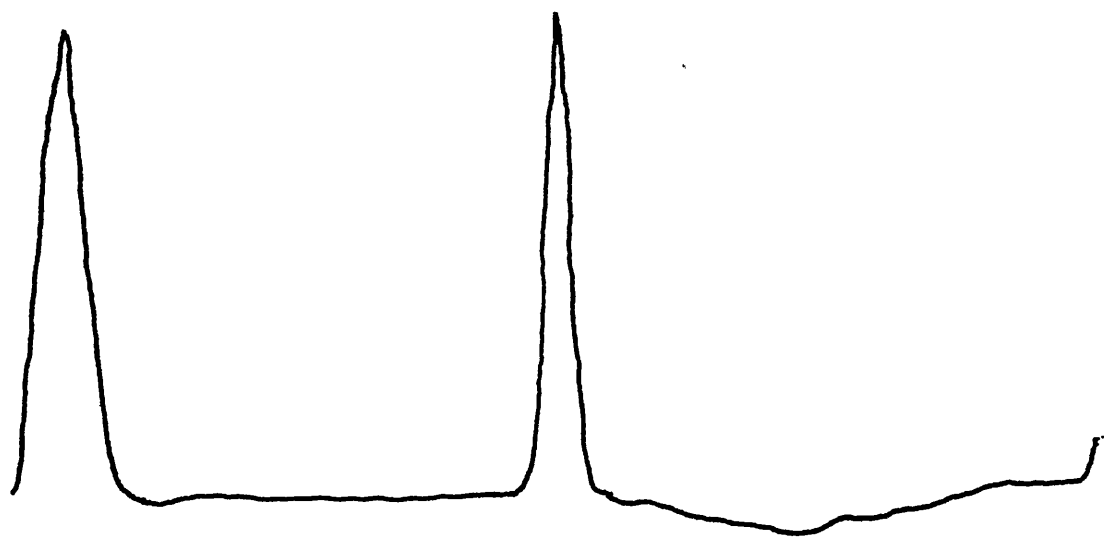


Electrophoresis at pH6.7 and staining were performed as described in Methods.

(A) Untreated enzyme, active prior to electrophoresis.

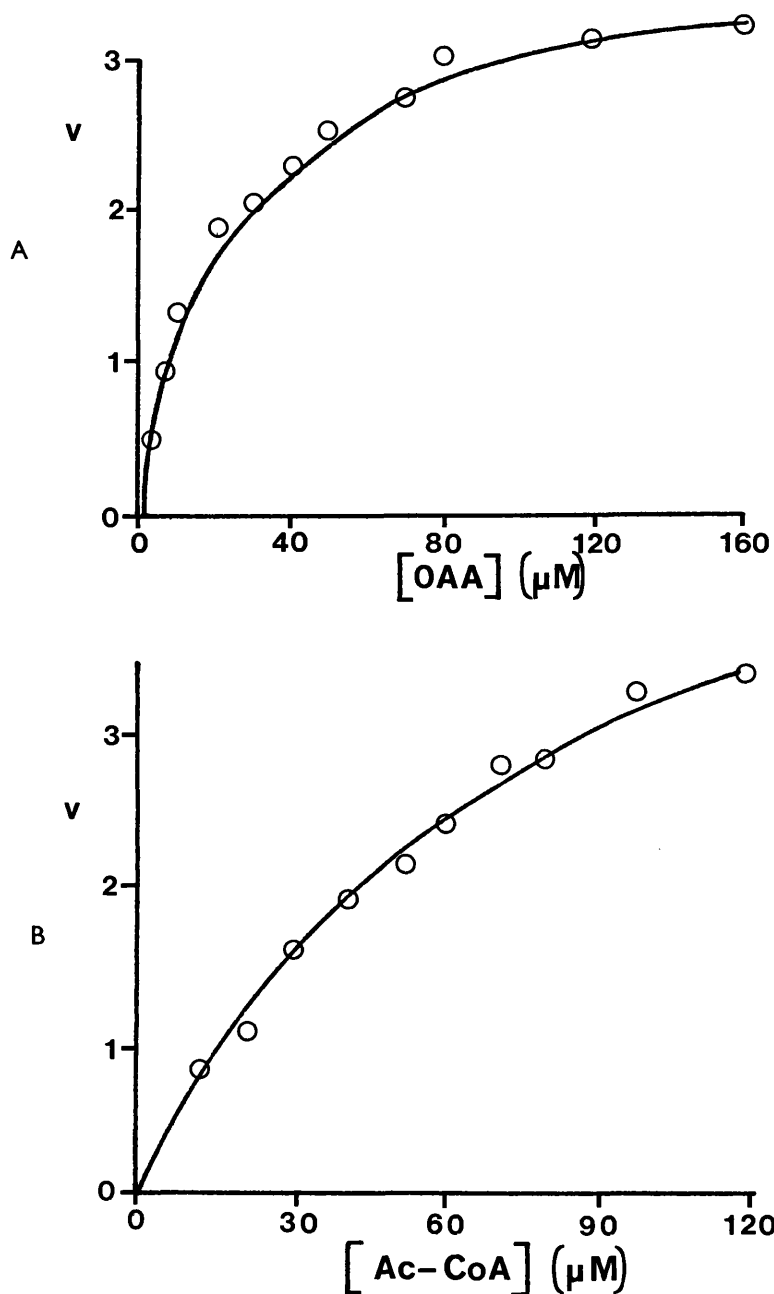
(B) Enzyme rendered inactive by treatment with hydroxylamine prior to electrophoresis.

1. The first peak is at $2\theta = 20^\circ$ and the second peak is at $2\theta = 25^\circ$.
 The third peak is at $2\theta = 30^\circ$ and the fourth peak is at $2\theta = 35^\circ$.



The first peak is at $2\theta = 20^\circ$ and the second peak is at $2\theta = 25^\circ$.
 The third peak is at $2\theta = 30^\circ$ and the fourth peak is at $2\theta = 35^\circ$.

Figure 42. Relationship of Reaction Velocity (v) versus $[S]$ for
Citrate Synthase from *B. megaterium* at pH8.0

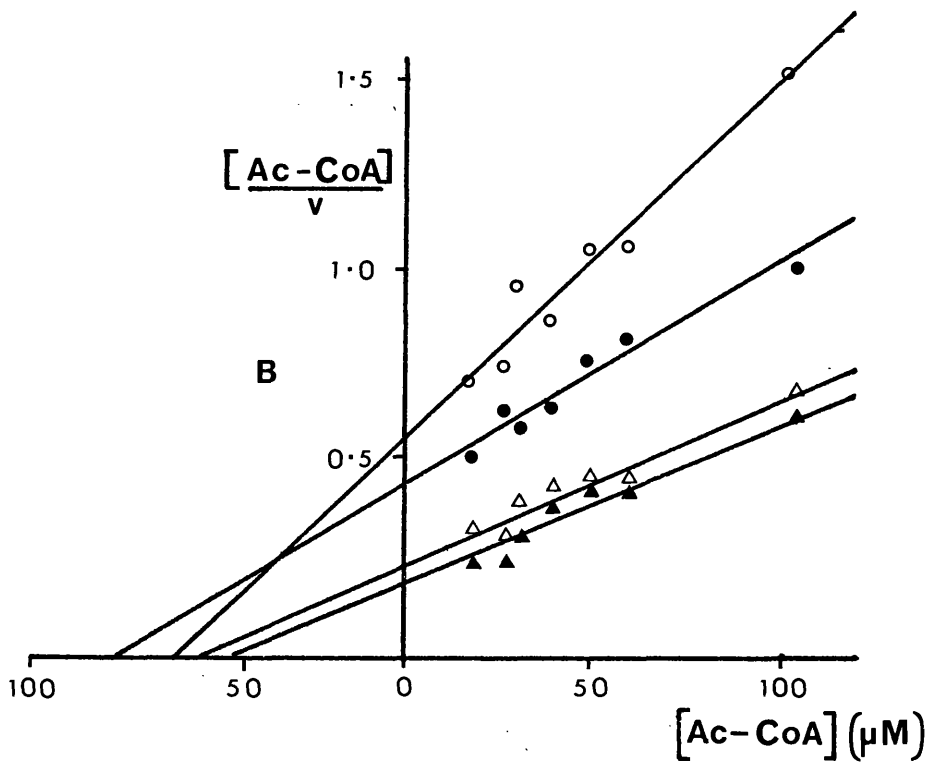
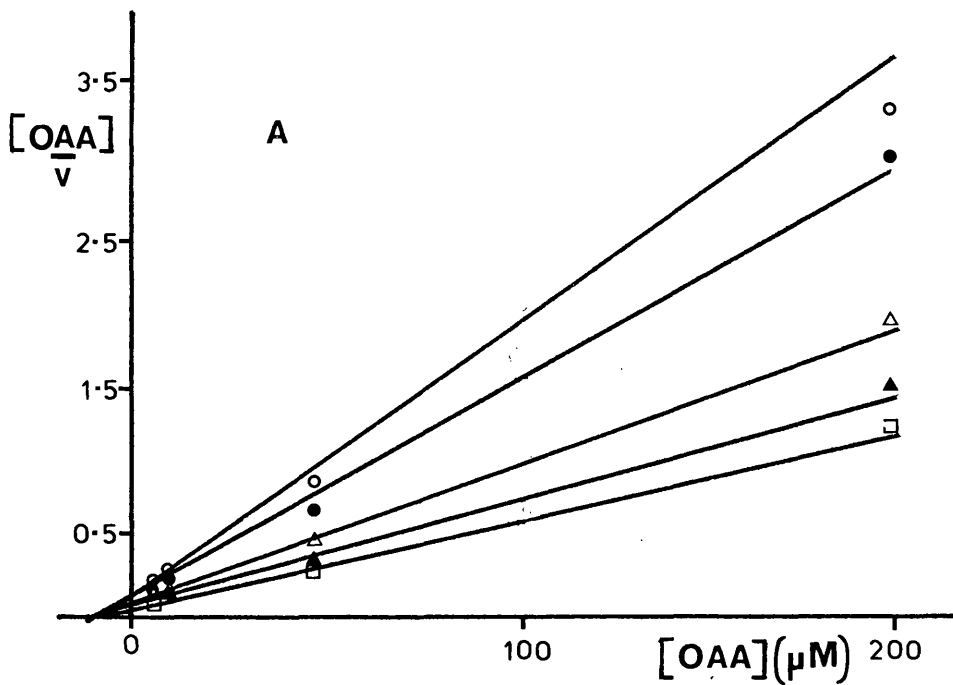


Enzyme assays were performed in 'Tris buffer B' at 412nm. v is measured in arbitrary units.

(A) v versus [OAA] at a fixed concentration of acetyl-CoA of 150 μM .

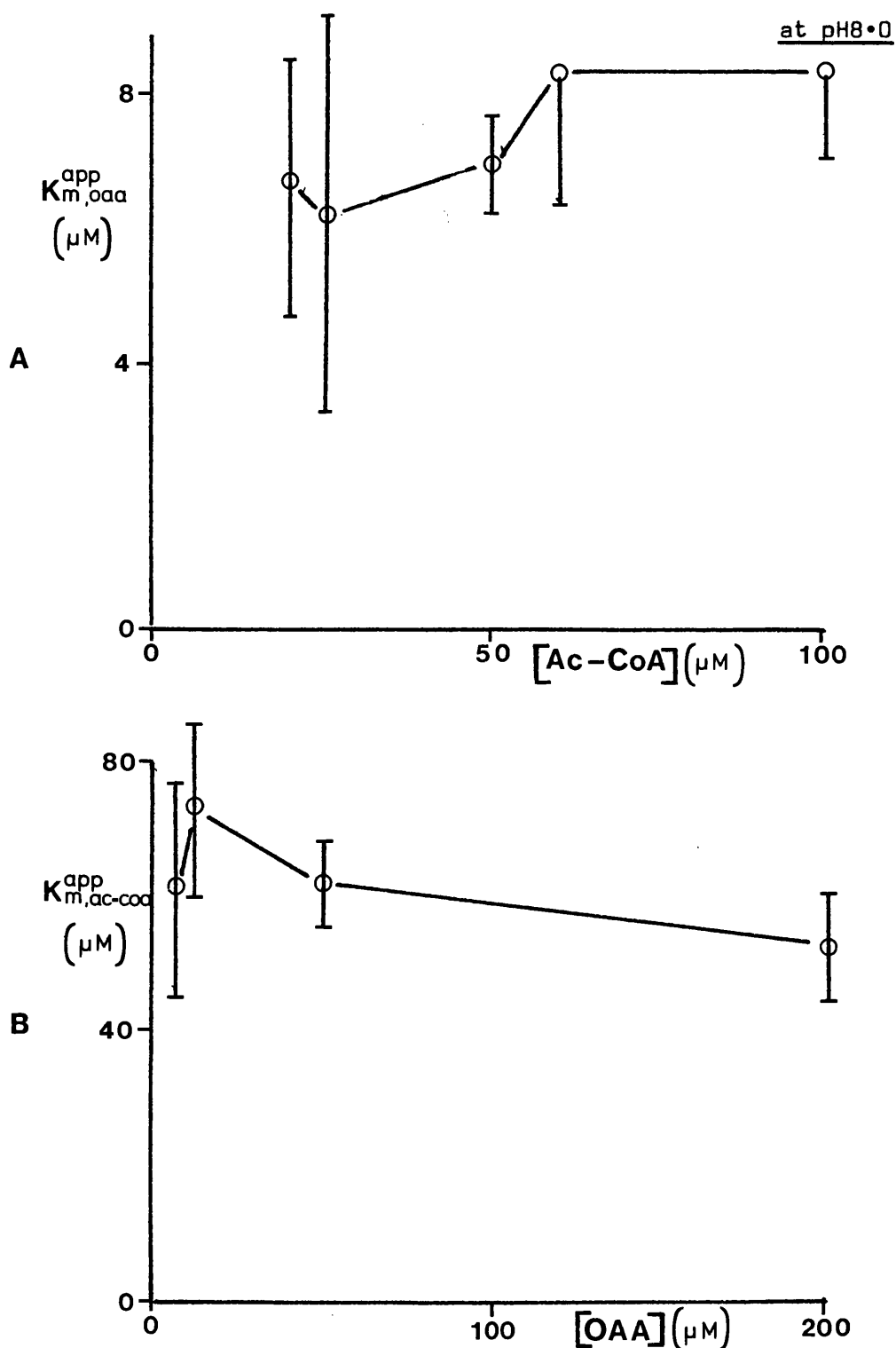
(B) v versus [acetyl-CoA] at a fixed concentration of oxaloacetate of 200 μM .

Figure 43. Hanes-Woolf Plots: $[S]/v$ versus $[S]$ for Citrate Synthase
from *B. megaterium* at pH8.0



Assays were performed in 'Tris buffer B' at 412nm.
 (a) Determination of the K_m for oxaloacetate at various fixed concentrations of acetyl-CoA. Concentrations of acetyl-CoA were: (○), 20 μM ; (●), 25 μM ; (△), 50 μM ; (▲), 60 μM ; and (□), 100 μM .
 (b) Determination of the K_m for acetyl-CoA at various fixed concentrations of oxaloacetate. Concentrations of oxaloacetate were: (○), 5 μM ; (●), 10 μM ; (△), 50 μM and (▲), 200 μM .

Figure 44. Kinetic Behaviour of Citrate Synthase from *B. megaterium*



(a) Dependence of the K_m^{app} for oxaloacetate on the concentration of acetyl-CoA.

(b) Dependence of the K_m^{app} for acetyl-CoA on the concentration of oxaloacetate.

Values for K_m^{app} and (S.E.M., represented by bars) were determined from figure 43.

$$\frac{[S]}{V_{\max}^{\text{app}}} \quad \text{versus} \quad [S] \quad (\text{figure 45}).$$

The K_m for oxaloacetate at infinite acetyl-CoA concentration was determined to be $9.2 (\pm 2.5) \mu\text{M}$ and the K_m for acetyl-CoA at infinite oxaloacetate concentration was determined to be $87.9 (\pm 37.9) \mu\text{M}$.

Inhibition by a range of adenine nucleotides was studied and the results are presented in the form of a histogram (figure 46). The order of effectiveness of inhibition was $\text{ATP} > \text{ADP} > \text{AMP}$. When the experiment with ATP was repeated in the presence of 10mM MgCl_2 , inhibition by ATP was significantly reduced. A similar study was carried out using a range of related nicotinamide adenine dinucleotides and the results are presented in figure 47. The order of effectiveness of inhibition in this case was $\text{NADPH} > \text{NADP}^+ > \text{NADH} > \text{NAD}^+$.

A more detailed study of the inhibition by ATP was carried out using various concentrations of acetyl-CoA over a range of fixed ATP concentrations. The results are presented as a series of plots of the form: $\frac{1}{v}$ versus $\frac{1}{[S]}$ (figure 48). ATP behaved as a

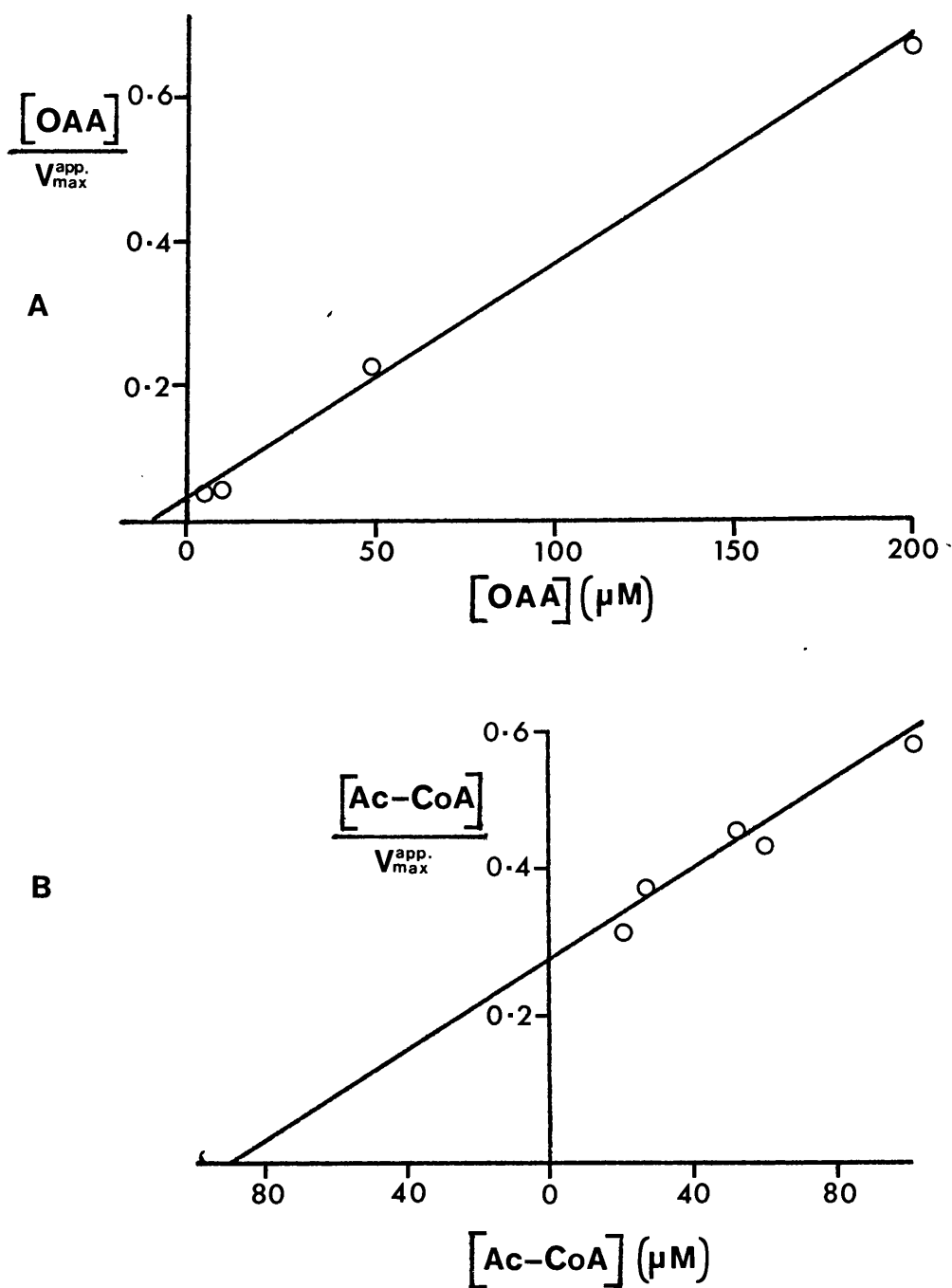
competitive inhibitor with respect to acetyl-CoA, the K_m^{app} for acetyl-CoA rising with increasing ATP concentrations and V_{\max}^{app} remaining unchanged. When secondary plots of $\frac{K_m^{\text{app}}}{V_{\max}^{\text{app}}}$ versus ATP

concentration were constructed, the K_i for ATP was determined to be $1.1 (\pm 0.1) \text{mM}$ (figure 49).

(b) At pH 6.5

In the light of the results obtained using inactivation by DEPC of citrate synthase at pH 6.5, it was decided to investigate the kinetic properties of B. megaterium citrate synthase under the same conditions.

Figure 45. Secondary Plots of $[S] / V_{\max}^{\text{app}}$ versus $[S]$ for Citrate Synthase from *B. megaterium* at pH8.0

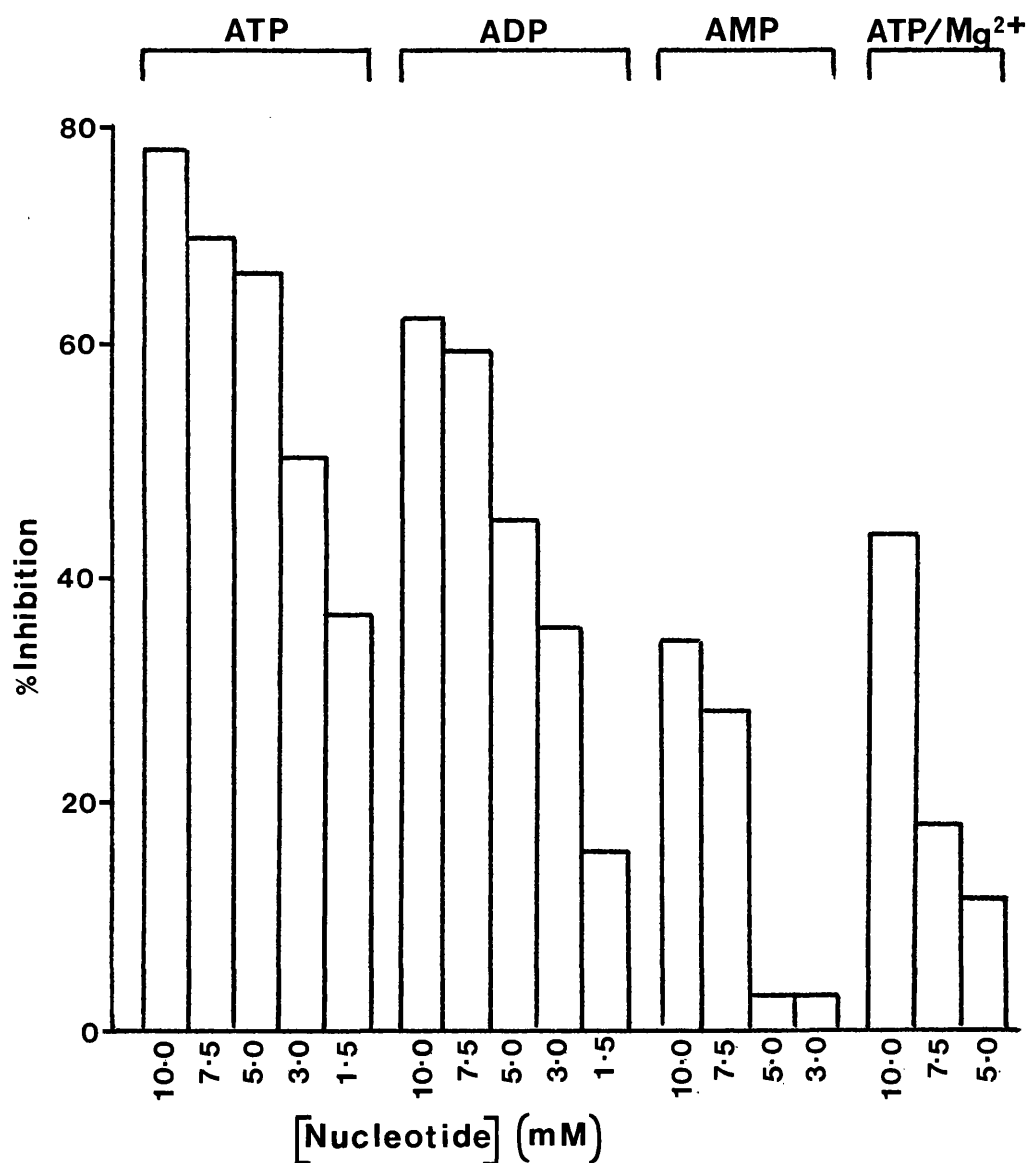


The values for V_{\max}^{app} at the various concentrations of substrate were determined from figure 43.

(a) Determination of the K_m for oxaloacetate at an infinite concentration of acetyl-CoA.

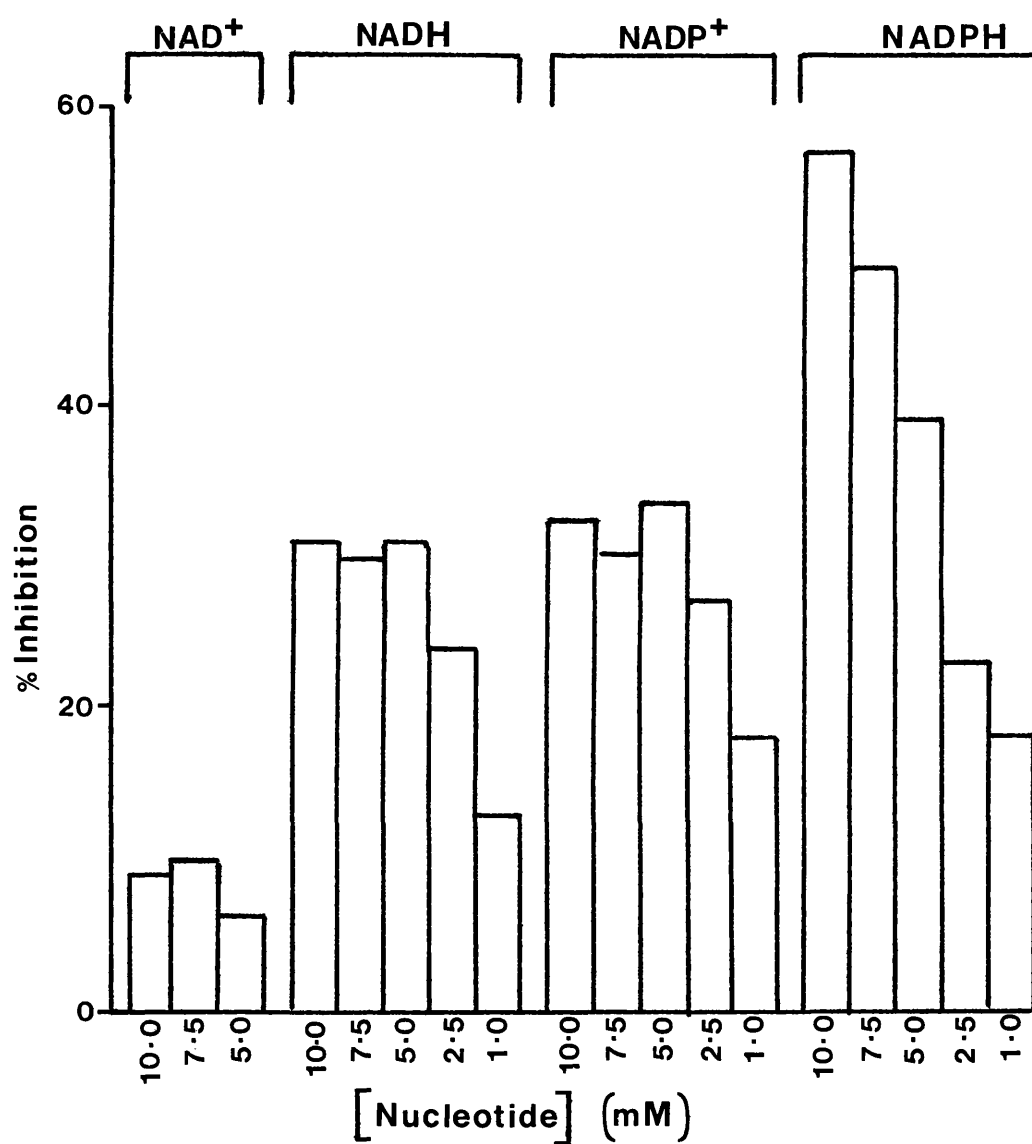
(b) Determination of the K_m for acetyl-CoA at an infinite concentration of oxaloacetate.

Figure 46. Inhibition by Adenine Nucleotides of Citrate Synthase
from *B. megaterium*



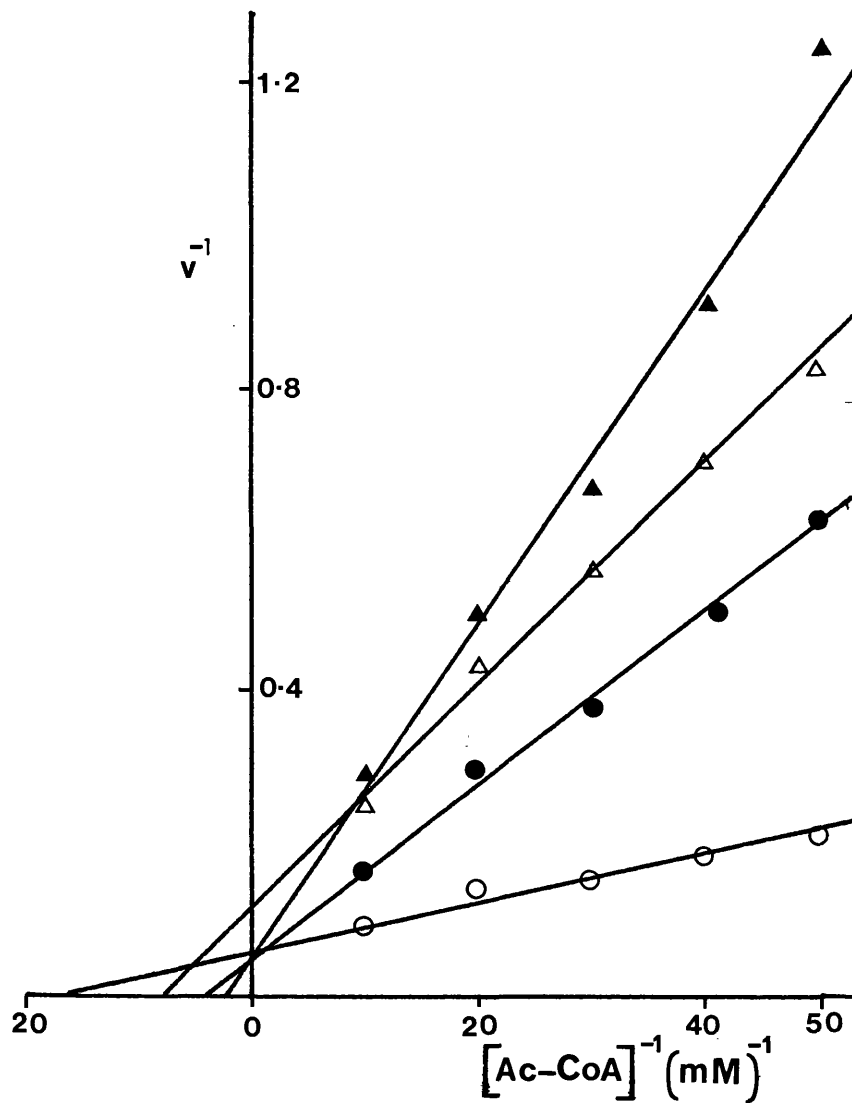
Citrate synthase was assayed at 412nm in 'Tris buffer C' containing acetyl-CoA (150μM), oxaloacetate (200μM) and DTNB (100μM), as described in Methods.

Figure 47. Inhibition by Nicotinamide Adenine Dinucleotides of
Citrate Synthase from *B. megaterium*



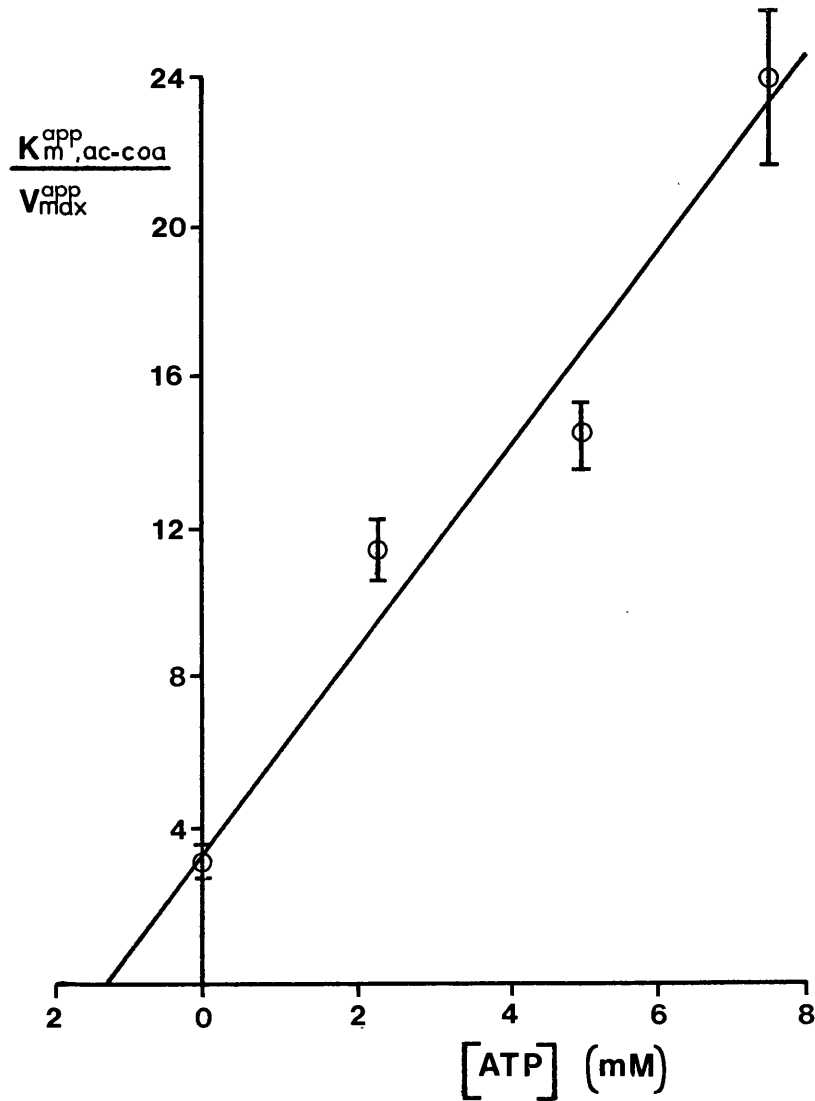
Citrate synthase was assayed at 412nm in 'Tris buffer C' containing acetyl-CoA (150μM), oxaloacetate (200μM) and DTNB (100μM), as described in Methods.

Figure 48. Double Reciprocal Plot of $1/v$ versus $1/[Acetyl-CoA]$ at Various fixed concentrations of ATP for Citrate Synthase from *B. megaterium* at pH8.0



The assays were performed in 'Tris buffer C' at 412nm in the presence of 200 M oxaloacetate. Concentrations of ATP were (○), 0mM; (●), 2.5mM; (△), 5.0mM; and (▲), 7.5mM.

Figure 49 Secondary Plots of K_m^{app} , Acetyl-CoA/ $V_{\text{max}}^{\text{app}}$ versus $[\text{ATP}]$
for the Determination of the K_i for ATP, at pH8.0,
for Citrate Synthase from *B. megaterium*



Values for K_m^{app} acetyl-CoA, $V_{\text{max}}^{\text{app}}$, (and S.E.M., represented by bars) were determined from figure 48.

The assays were performed in 50mM sodium acetate buffer, pH6.5, containing 1mM EDTA and 20% (v/v) glycerol. The decrease in absorbance was followed at 232 nm, accompanying the cleavage of the S-acyl bond in acetyl-CoA (Srere and Kosicki, 1961). The spectrophotometric assay at 412nm using DTNB is unsuitable at pH6.5 for two reasons. First, the product TNBS⁻ is protonated at pH6.5 and is thus rendered colourless. Second, the thiol group of CoA is also protonated at pH6.5 and will not carry out nucleophilic attack on the disulphide bond of DTNB to produce TNBS⁻.

The enzyme was assayed using 5 fixed concentrations of oxaloacetate with a range of 5 different concentrations of acetyl-CoA. The results are presented in the form: $\frac{[S]}{v}$ versus $[S]$ (figure 50).

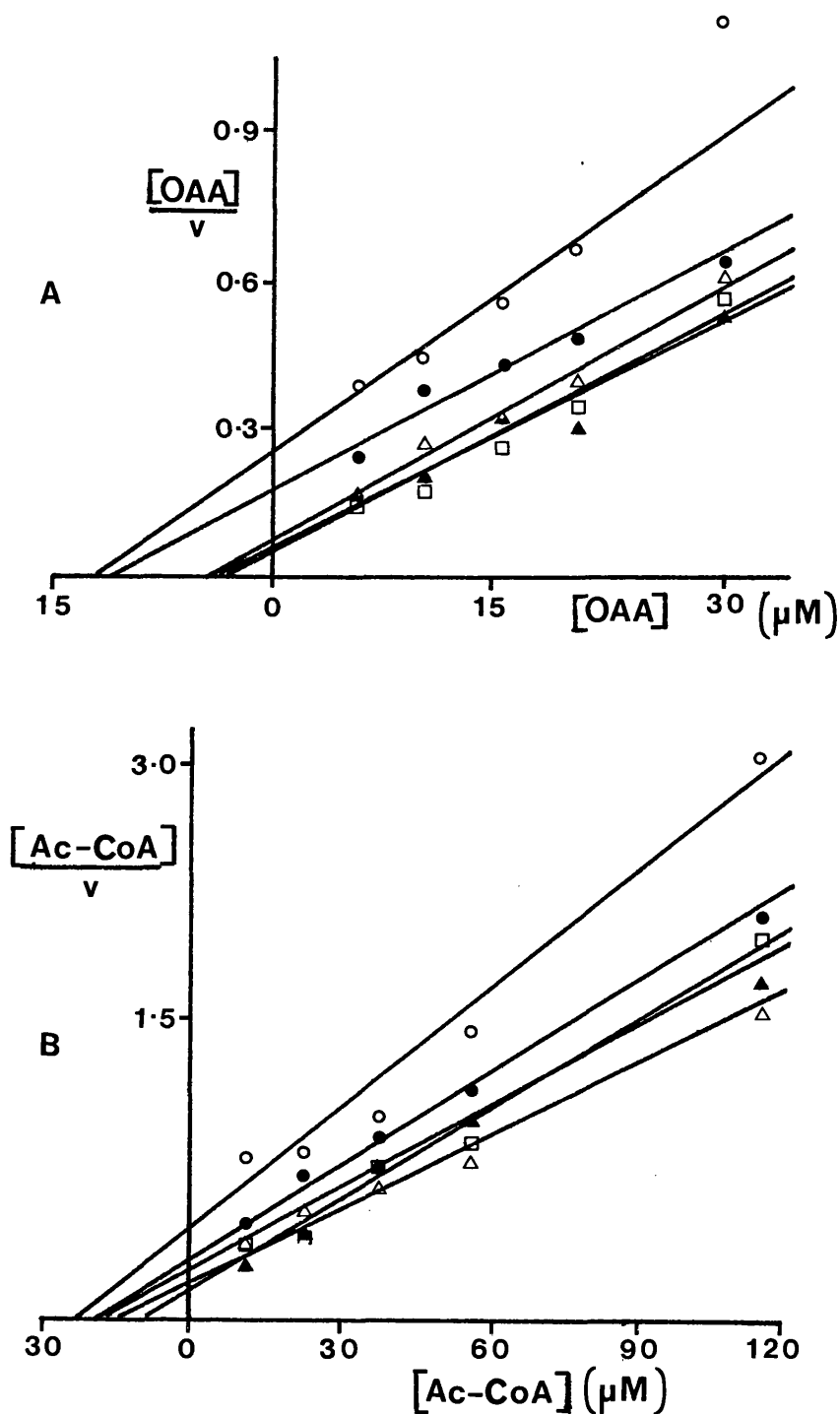
Both substrates appeared to follow Michaelis-Menten kinetics at each fixed concentration of the other. However the K_m^{app} for oxaloacetate appeared to decrease with increasing concentrations of acetyl-CoA (figure 51a) and similarly the K_m^{app} for acetyl-CoA decreased with increasing oxaloacetate concentration (figure 51b).

The Michaelis-Menten equation may be modified in the case of a two substrate reaction (Wharton and Eisenthal, 1981). In this case keeping the concentration of acetyl-CoA fixed and varying that of oxaloacetate.

$$v_{max}^{app} = \frac{v_{max} [Ac-CoA]}{[Ac-CoA] + K_m^{Ac-CoA}} \quad (3)$$

where K_m^{Ac-CoA} is the Michaelis constant for acetyl-CoA at an infinite concentration of oxaloacetate.

Figure 50. Hanes-Woolf plots: $[S]/v$ versus $[S]$ for Citrate Synthase from *B. megaterium* at pH6.5



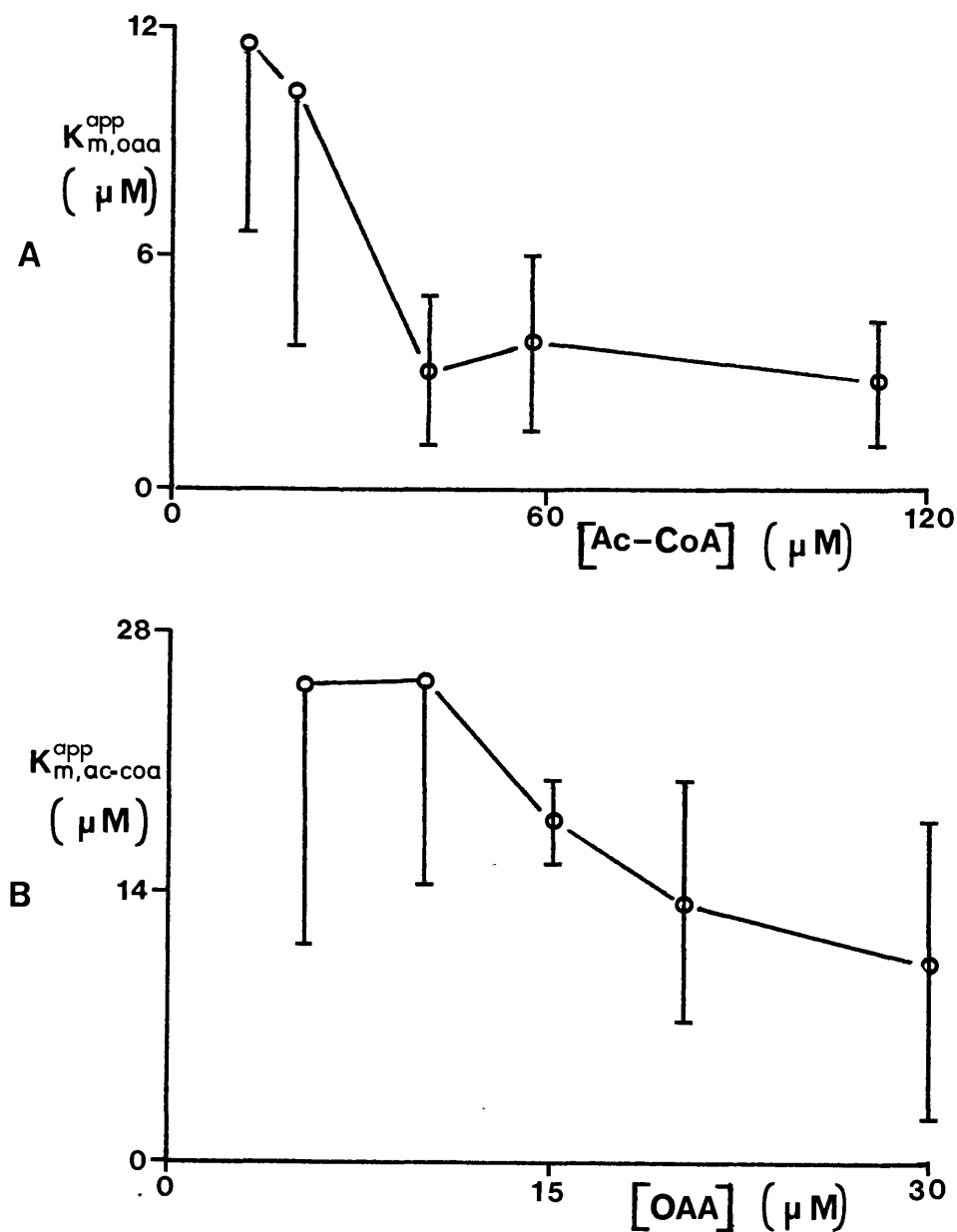
Assays were performed in acetate buffer at 232nm.

(a) Determination of K_m^{app} for oxaloacetate at various fixed concentrations of acetyl-CoA. Concentrations of acetyl-CoA were: (O), 11.3 μM ; (●), 18.8 μM ; (Δ), 37.5 μM ; (\blacktriangle), 56.3 μM ; and (\square), 112.5 μM .

(b) Determination of K_m^{app} for acetyl-CoA at various fixed concentrations of oxaloacetate. Concentrations of oxaloacetate were: (O), 5 μM ; (●), 10 μM ; (Δ), 15 μM ; (\blacktriangle), 20 μM ; and (\square), 30 μM .

Figure 51. Kinetic Behaviour of Citrate Synthase from *B. megaterium*

at pH6.5



(a) Dependence of the K_m^{app} for oxaloacetate on the concentration of acetyl-CoA.

(b) Dependence of the K_m^{app} for acetyl-CoA on the concentration of oxaloacetate.

The values for K_m^{app} (and S.E.M., represented by bars) for both substrates were determined from figure 50.

Also

$$\frac{V_{\max}^{\text{app}}}{K_{m \text{ app}}^{\text{OAA}}} = \frac{V_{\max} [\text{Ac-CoA}] / K_m^{\text{OAA}}}{[\text{Ac-CoA}] + K_s^{\text{OAA}} \cdot K_m^{\text{Ac-CoA}} / K_m^{\text{OAA}}} \quad (4)$$

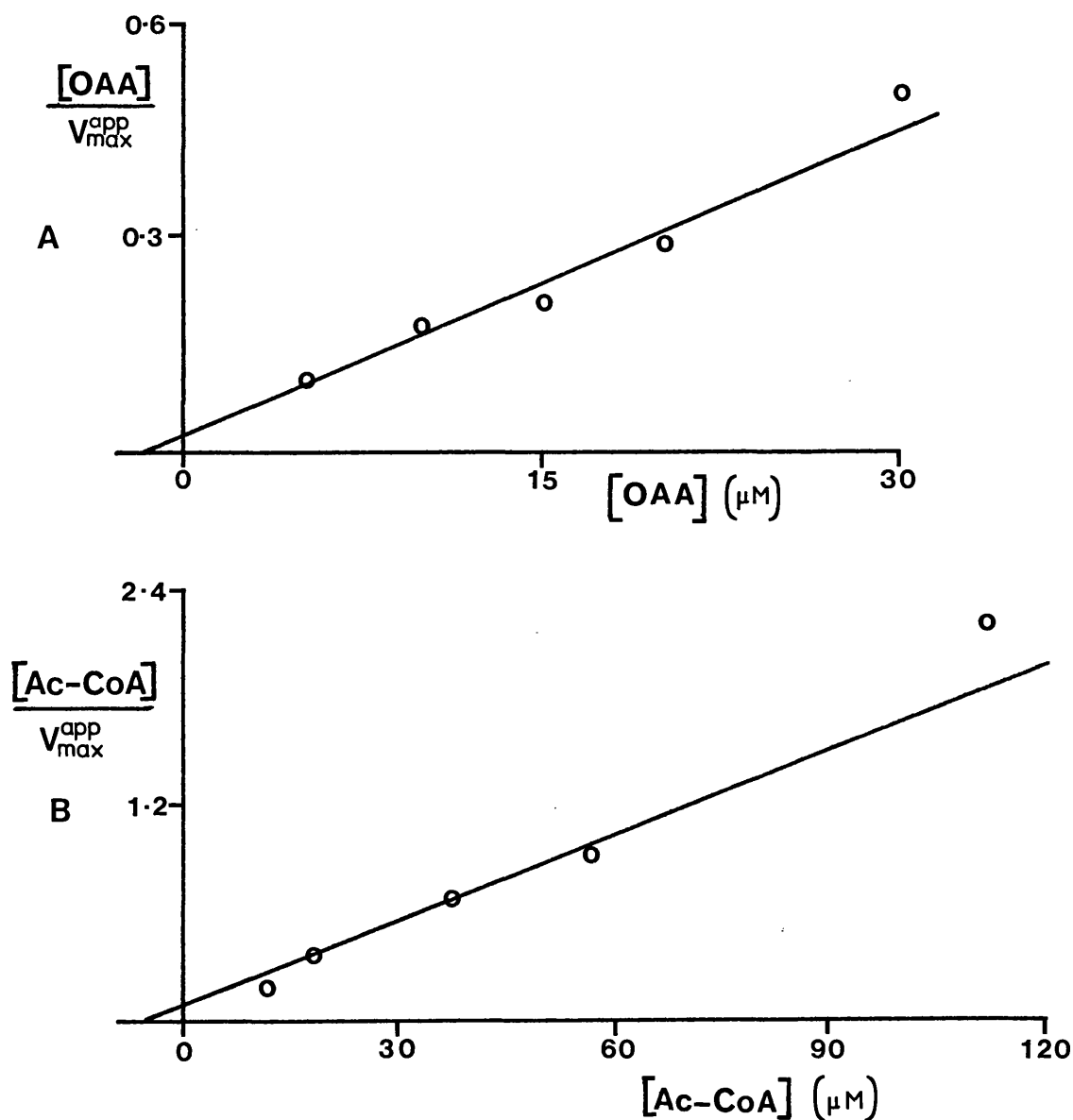
where K_s^{OAA} can be considered the dissociation constant of the enzyme substrate complex (K_D^{OAA}) provided that there are no intermediate steps (eg isomerization) between the binding of oxaloacetate and acetyl-CoA to citrate synthase. Thus, the values of V_{\max} and $K_m^{\text{Ac-CoA}}$ can be obtained from equation 3 by a direct linear plot of V_{\max}^{app} versus $[\text{Ac-CoA}]$. Similarly $V_{\max} / K_m^{\text{OAA}}$ and $K_s^{\text{OAA}} \cdot K_m^{\text{Ac-CoA}} / K_m^{\text{OAA}}$ can be obtained from equation 4 by a direct linear plot of $V_{\max}^{\text{app}} / K_{m \text{ app}}^{\text{OAA}}$ versus $[\text{Ac-CoA}]$, and using the values determined for $K_m^{\text{Ac-CoA}}$ and K_m^{OAA} the calculation of K_s^{OAA} can be performed. A similar treatment may be applied considering oxaloacetate to be at fixed concentrations and varying concentrations of acetyl-CoA, thus allowing the determination of $K_s^{\text{Ac-CoA}}$. These secondary plots were constructed and are shown in figures 52 and 53. The K_m^{OAA} was determined to be $2(\pm 1)\mu\text{M}$ and the $K_m^{\text{Ac-CoA}}$ was calculated to be $6(\pm 2)\mu\text{M}$. The value for the K_s^{OAA} was determined to be $10(\pm 4)\mu\text{M}$ and $K_s^{\text{Ac-CoA}}$ was calculated to be $83(\pm 27)\mu\text{M}$.

Discussion

Purification

This chapter describes the first reported purification to homogeneity of the citrate synthase from B. megaterium. In the purification procedure affinity chromatography using Sepharose-ATP was attempted. This involves the specific elution of citrate synthase

Figure 52. Secondary Plots of $[S]/v_{\max}^{\text{app}}$ versus $[S]$ for Citrate Synthase from *B. megaterium* at pH6.5

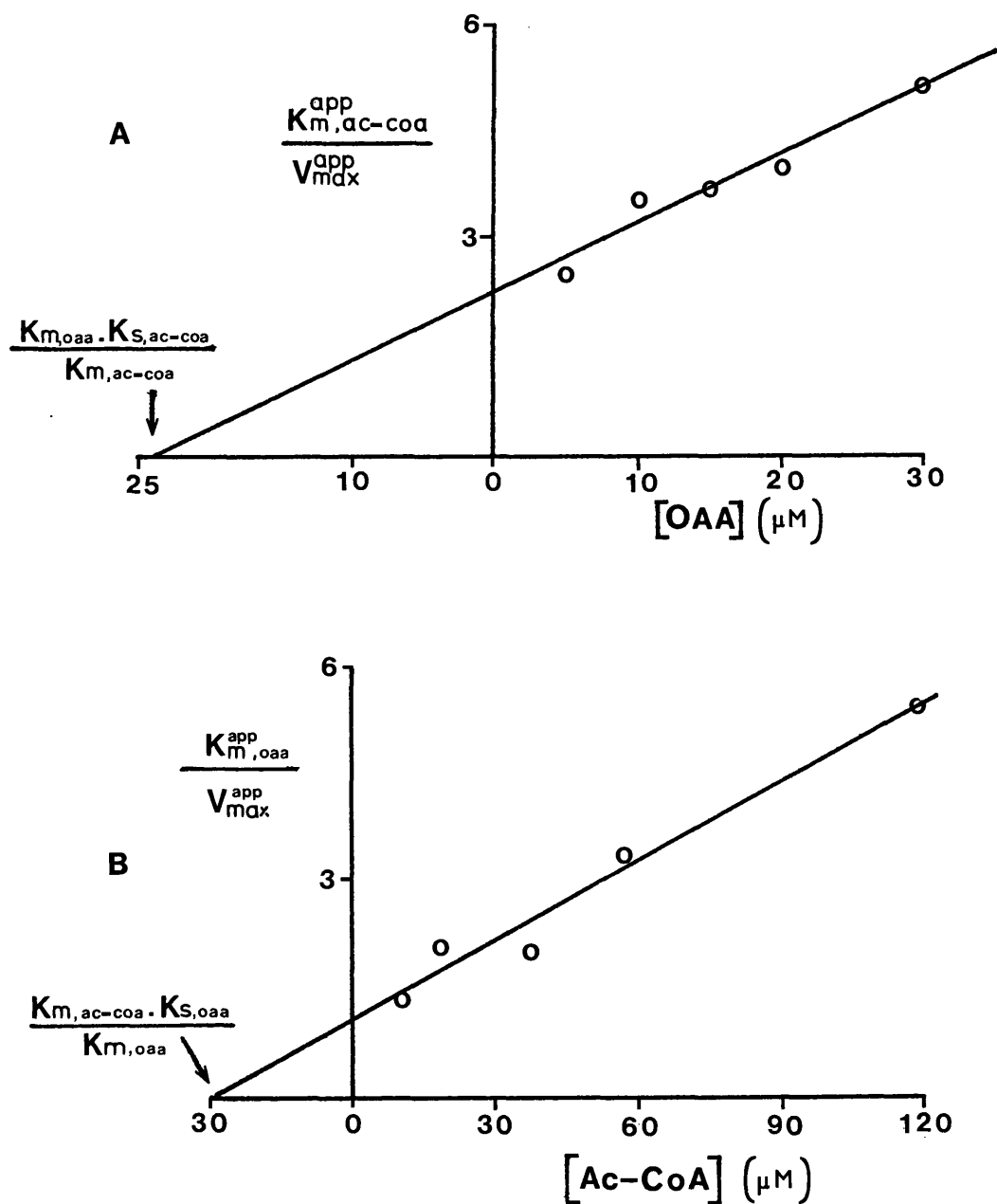


The values for v_{\max}^{app} at the various concentrations of substrate were determined from figure 50.

(a) Determination of the K_m for oxaloacetate at an infinite concentration of acetyl-CoA.

(b) Determination of the K_m for acetyl-CoA at an infinite concentration of oxaloacetate.

Figure 53. Secondary Plots of K_m^{app} versus $[S]$ for Citrate Synthase
 Synthase from *B. megaterium* at pH6.5.



Values for K_m^{app} and $V_{\text{max}}^{\text{app}}$ were obtained from figure 50. Values for K_m were determined from figure 52.

(a) Determination of the K_s for acetyl-CoA.

(b) Determination of the K_s for oxaloacetate.

from Sepharose-ATP with the dead end complex-forming substrates, oxaloacetate and CoA, and has been achieved successfully with citrate synthase from eukaryotic sources (Mukherjee and Srere, 1976). However, B. megaterium citrate synthase did not bind to Sepharose-ATP and this may be a reflection of the K_i for ATP (1mM) compared to the corresponding value for the enzyme from eukaryotic sources (0.1mM - 0.5mM) (Srere et al., 1973; Shepherd and Garland, 1969).

M_r of Native Enzyme and Subunits

The enzyme was determined to have an M_r value of 84,000 and was dimeric with subunits of equal molecular size. It therefore resembles the enzyme from eukaryotic sources in this respect.

The results obtained by sedimentation-equilibrium analysis, indicating the presence of more than one molecular species, suggest that the enzyme may dissociate under prolonged periods of centrifugation. In contrast the enzyme behaved as a single molecular species in sedimentation-velocity analysis and remained stable when stored under the same conditions in which sedimentation-equilibrium ultracentrifugation was performed.

Modification by Specific Chemical Reagents

B. megaterium citrate synthase was insensitive to DTNB, a reagent specific for thiol groups. This suggests that these groups may be buried in the interior of the enzyme and are therefore unavailable for modification.

The enzyme was inactivated by DEPC, a reagent specifically modifying histidine residues. Spectroscopic analysis of the modified citrate synthase indicated that 2 histidine residues per enzyme molecule underwent modification with 100% loss in catalytic activity. If the subunits are identical then it is likely that 1 histidine residue per subunit is sensitive to DEPC.

Both substrates and ATP offered protection against enzyme-inactivation by DEPC suggesting that the histidine residue is at or very near the active site. The observation that only oxaloacetate fully protects against inactivation indicates that this histidine residue may be involved in binding oxaloacetate to B. megaterium citrate synthase.

Kinetic Studies and Dissociation Constants of Substrates

A summary of the kinetic constants and dissociation constants obtained under various conditions is shown in table 7.

At pH6.5 the K_m for each substrate was lower than at pH8.0, particularly the value for acetyl-CoA which was decreased by a factor of approximately 15-fold. The dependence of the K_m of each substrate on the concentration of the other at pH6.5 indicates that the enzyme responds to its substrates in a different manner from that at pH8.0. This may be due either to conformational differences in the enzyme at pH8.0 and 6.5; or to charge differences in various functional groups between these two pH values.

The K_D values obtained at pH6.5 using inactivation studies with DEPC suggest that either oxaloacetate or acetyl-CoA binds to the free enzyme. Whether the reaction is a sequential random order or a sequential preferred order can be determined only by product-inhibition studies.

Table 7. Comparison of the Kinetic Constants and Dissociation
Constants of *B. megaterium* Citrate Synthase for
Substrates and ATP

Ligand	At pH8.0 K_m of substrate or K_i of ATP (μM)	K_m (μM)	At pH6.5 K_s (μM)	K_D (μM)
Oxaloacetate	9 (± 3)	2 (± 1)	10 (± 4)	2 (± 0.2)
Acetyl-CoA	88 (± 38)	6 (± 2)	83 (± 27)	51 (± 5)
ATP	1100 (± 100)	-	-	28 (± 5)

CHAPTER 4

The Citrate Synthase from *E. coli* K114R

The Citrate Synthase from *E. coli* K114R

Introduction

E. coli K114R possesses a mutant citrate synthase which was generated in the laboratory by selecting, from a citrate synthase-deficient mutant of *E. coli*, revertants containing functional but variant citrate synthases (Danson *et al.*, 1979b). Several such revertants were isolated and categorised. The citrate synthase from *E. coli* K114R was observed to be of the 'small' type, and was insensitive to both NADH and 2-oxoglutarate. It was however inhibited by ATP and displayed hyperbolic dependences on both acetyl-CoA and oxaloacetate. The enzyme therefore appears to resemble closely the citrate synthases from eukaryotes and Gram-positive bacteria.

No detailed structural studies on this mutant citrate synthase have yet been performed, as its purification is hampered by the low specific activity observed in crude extracts. The enzyme may also be less stable by virtue of the ease of oxidation of its thiol groups, since it underwent very rapid inactivation by DTNB (Danson *et al.*, 1979b).

An attempt was made to purify this enzyme in order to undertake more detailed comparisons with the 'wild-type' *E. coli* 'large' citrate synthase and the 'small' enzymes from eukaryotes and Gram-positive bacteria.

Growth Characteristics of *E. coli* K114R

Before proceeding with the purification and characterisation

of the citrate synthase from E. coli K114R it was necessary to verify that the organism still retained the growth characteristics described by Danson et al. (1979b) namely its requirement for both methionine and thymine and the characteristics of E. coli.

(a) Tests for E. coli

When exposed to Gram-staining procedure followed by microscopic examination the bacteria appeared as Gram-negative rods. Like wild-type E. coli no growth was observed on citrate as sole carbon source. After growth on Tryptone broth (10g/l) for 3 days at 44°C indole production was detected by Ehrlich's reagent, 4-dimethylaminobenzaldehyde, by the production of a rose-pink colour. The organism grew as dark red colonies on MacConkey agar.

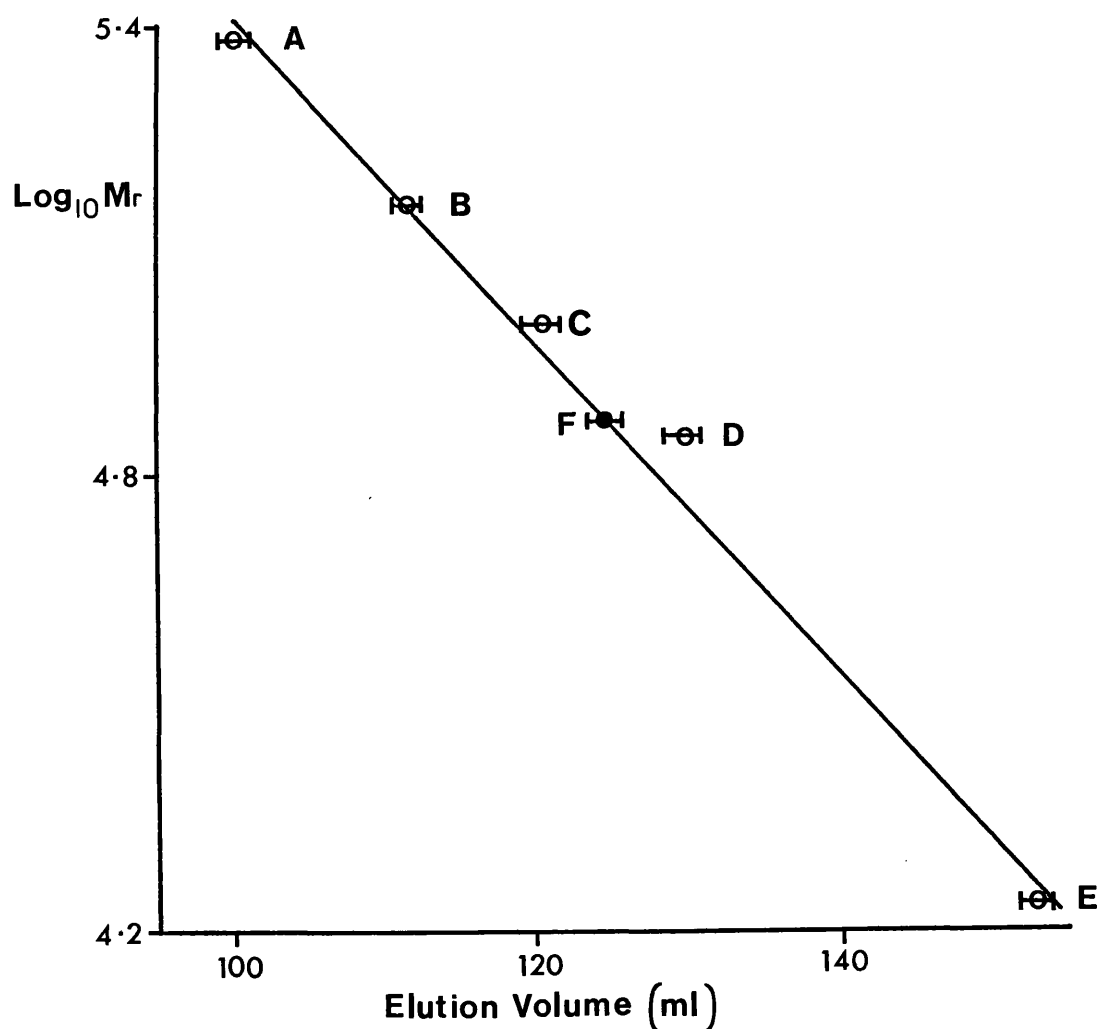
(b) Tests for K114R

The organism grew on defined media described by Ashworth and Kornberg (1966) but had an absolute requirement for both methionine (20mg/l) and thymine (20mg/l) and was not auxotrophic for glutamate. It was concluded from these results that the organism was E. coli and had the same growth requirements as those originally described by Danson et al. (1979b).

The Molecular Size of Citrate Synthase from E. coli K114R

The mutant citrate synthase was found to have a tendency to revert to the wild-type form and E. coli possessing the wild-type citrate synthase has the ability to outgrow E. coli K114R. It was therefore necessary to verify that E. coli K114R still retained the 'small' citrate synthase reported by Danson et al. (1979b).

Figure 54. Gel Filtration on Sephacryl S-220 - Relationship
between $\log_{10} M_r$ and Elution Volume.



Gel filtration in Tris buffer C was performed as described in Methods. Proteins used as standards were: (A), bovine liver catalase (M_r 244,000); (B), pig heart lactate dehydrogenase (M_r 144,000); (C), pig heart citrate synthase (M_r 98,000); (D), pig heart malate dehydrogenase (M_r 70,000); and (E), whale skeletal-muscle myoglobin (M_r 17,200). (F) represents the citrate synthase from *E. coli* K114R. The bars represent the S.E.M. of the volumes at which each protein was eluted in five separate experiments.

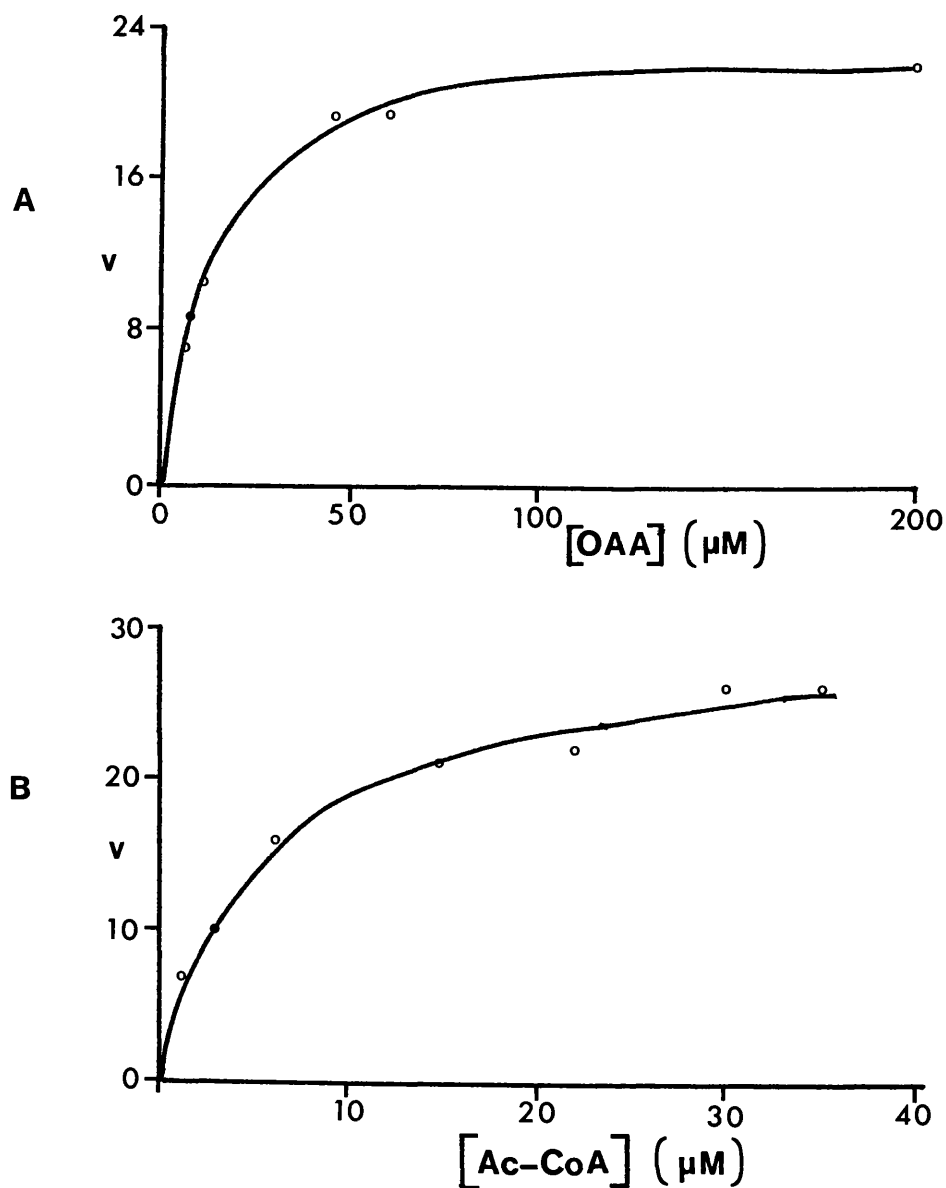
The M_r of the enzyme was determined using zonal filtration on a column (40cmx2cm) of Sephacryl S-200 (superfine grade) in 20mM Tris/HCl buffer, pH8.0, containing 1mM EDTA hereafter known as 'Tris buffer C'. A flow rate of 10ml/h was used and fractions (0.8ml) were collected. Proteins used as standards were: beef liver catalase, pig heart lactate dehydrogenase, pig heart malate dehydrogenase, pig heart citrate synthase (run separately from the E. coli K114R enzyme) and whale-skeletal muscle myoglobin. The results were plotted as $\log_{10} M_r$ versus elution volume (ml) (figure 54). The citrate synthase from E. coli K114R eluted as a single symmetrical peak and the M_r thus determined was 76,000 ($\pm 1,000$).

Kinetic and Regulatory Properties

Following partial purification of the enzyme by ammonium sulphate fractionation and gel filtration the kinetic response to oxaloacetate and acetyl-CoA was studied in 'Tris buffer C' at a wavelength of 412nm as described in Methods. The K_m for oxaloacetate was determined at a concentration of 150 μ M acetyl-CoA and the K_m for acetyl-CoA was determined at a concentration of 200 μ M oxaloacetate. The enzyme displayed hyperbolic dependences on both substrates (figure 55). The results, analysed using the direct linear plot (Eisenthal and Cornish-Bowden, 1974) are presented as $[S]/v$ versus $[S]$ (figure 56). The K_m determined for oxaloacetate was 12(± 2) μ M and for acetyl-CoA was 4(± 1) μ M.

Inhibition by ATP was studied by determining K_m^{app} and V_{max}^{app} for acetyl-CoA at various fixed concentrations of ATP. The results, plotted as $[S]/v$ versus $[S]$ are shown in figure 57. There was no significant change in V_{max}^{app} and K_m^{app} for acetyl-CoA increased with

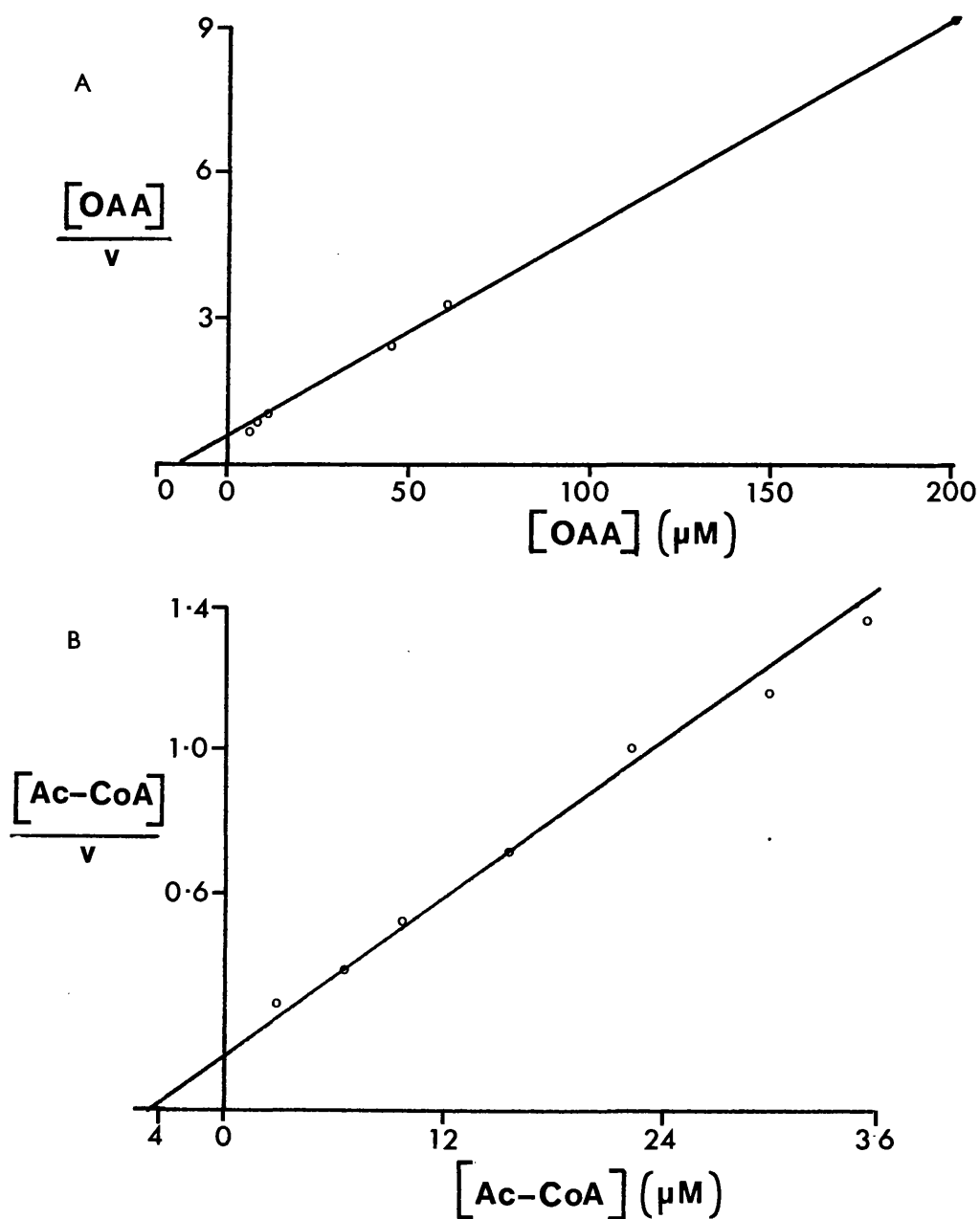
Figure 55. Relationship of Reaction Rate (v) Versus $[S]$ for
Citrate Synthase from *E. coli* K114R.



Enzyme assays were performed in 'Tris buffer C' at 412nm. v is measured in arbitrary units.

- (A) v versus $[OAA]$ at a fixed concentration of acetyl-CoA of $150\mu M$.
 (B) v versus $[Acetyl-CoA]$ at a fixed concentration of oxaloacetate of $200\mu M$.

Figure 56. Hanes-Woolf Plots: $[S]/v$ versus $[S]$ for Citrate Synthase from *E. coli* K114R

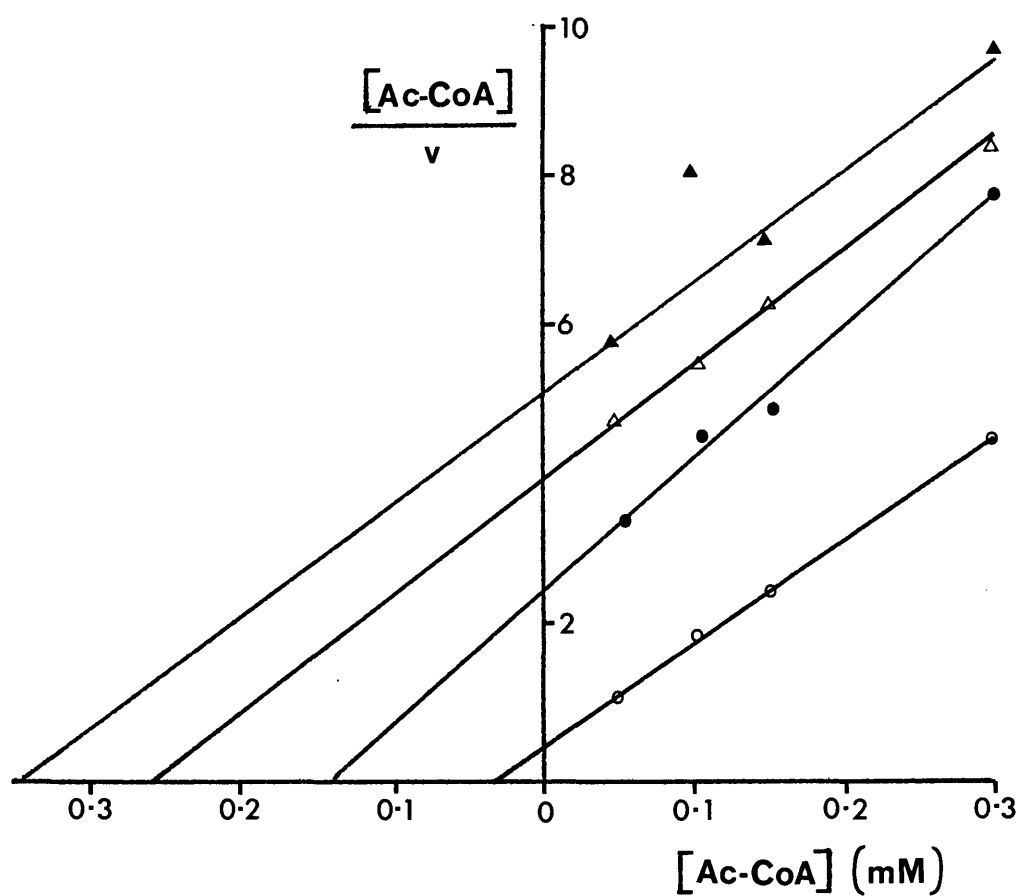


Assays were performed at 412nm in 'Tris buffer C'

- (a) Determination of the K_m for oxaloacetate at a fixed concentration of acetyl-CoA of 150 μM .
- (b) Determination of the K_m for acetyl-CoA at a fixed concentration of oxaloacetate of 200 μM .

Figure 57. Hanes-Woolf Plots of $[S]/v$ Versus $[S]$ for Acetyl-CoA
at Various Concentrations of ATP using *E. coli* K114R

Citrate Synthase



E. coli K114R citrate synthase was assayed in 'Tris buffer C' at 412nm as described in the text. Concentrations of ATP used were: (○), 0mM; (●), 4mM; (△), 6mM; and (▲), 8mM.

increasing ATP concentrations. These results suggest that ATP acts as a competitive inhibitor with respect to acetyl-CoA. A secondary plot of K_m^{app}/V_{max}^{app} versus ATP concentration was constructed (figure 58) and the K_i for ATP was determined as $205(\pm 30)\mu\text{M}$. Neither NADH ($200\mu\text{M}$) or 2-oxoglutarate ($200\mu\text{M}$) inhibited the citrate synthase from E. coli K114R.

Purification of E. coli K114R Citrate Synthase

An overnight (16h) culture at 37°C of 8l of Oxoid nutrient broth, supplemented with methionine (20mg/l) and thymine (20mg/l) yielded 14g of cells (wet weight) on harvesting. The cells were washed and resuspended in 'Tris buffer C' before being lysed in a French press and a cell-free extract prepared as described previously.

Nucleic acid was precipitated from this extract using protamine sulphate and the resulting supernatant was treated with ammonium sulphate to 40% saturation. After the precipitated protein had been removed by centrifugation most of the citrate synthase activity was found to remain in the supernatant. The ammonium sulphate saturation was increased to 60% and citrate synthase was recovered in the pellet after centrifugation and resuspension in 'Tris buffer C' (2ml).

The enzyme was subjected to gel filtration by application to a Sephadex G-200 column (35cmx3cm) previously equilibrated with 'Tris buffer C'. A flow rate of 10ml/h was used and fractions (2.5ml) were collected. Those fractions containing citrate synthase at a specific activity higher than 0.07 units/mg were pooled and applied to a Mono Q anion exchange column at room temperature using the

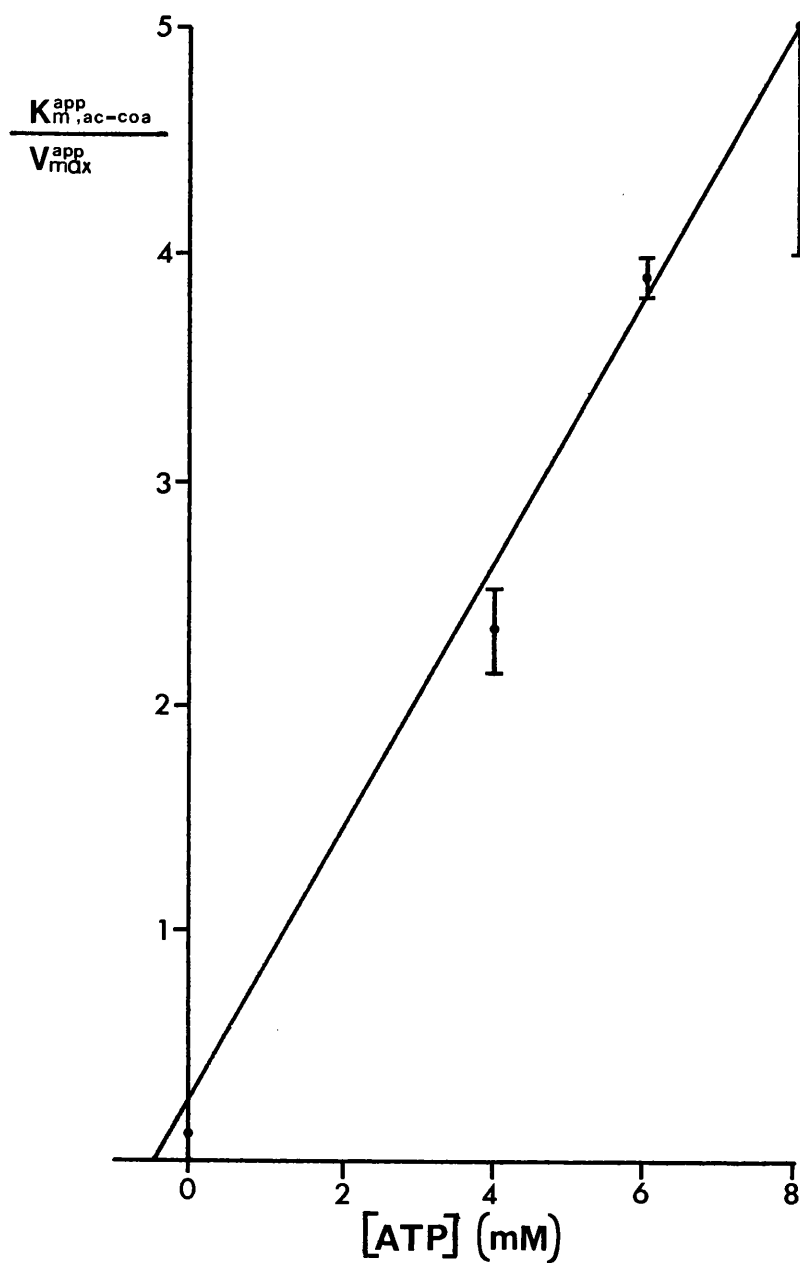


Figure 58. Secondary Plot of K_m^{app} , Acetyl Co-A/ V_{max}^{app} versus $[ATP]$
for the Determination of the K_i for ATP for Citrate
Synthase from *E. coli* K114R.

Values for K_m^{app} , acetyl-CoA, V_{max}^{app} (and S.E.M; represented by bars) were determined from figure 57.

Fast Protein Liquid Chromatography system (Pharmacia). The column had been previously equilibrated with 20mM triethanolamine/HCl buffer, pH7.3, containing 1mM EDTA. The column was washed with 5ml of this buffer and citrate synthase was eluted using a linear gradient of 0.2M - 0.4M sodium acetate in 52ml of buffer at a flow rate of 40ml/h. Fractions (1ml) were collected. Citrate synthase activity was detected in fractions containing acetate at concentrations of 0.31M - 0.37M. Those fractions containing citrate synthase with a specific activity higher than 0.7 units/mg were pooled and diluted 1:2 with triethanolamine/HCl, pH7.3, containing 1mM EDTA.

These pooled, diluted fractions were reapplied to the Mono Q column. The column was washed with 5ml of buffer and then a linear gradient of 0.2M - 0.4M sodium chloride was applied in 52ml of buffer at a flow rate of 40ml/l. The elution profile at 280nm is shown in figure 59. The fraction collector was programmed to collect those fractions having an absorbance at 280nm higher than 0.15. Citrate synthase activity was detected in fractions containing 0.24M - 0.26M sodium chloride.

Those fractions containing citrate synthase at a specific activity higher than 5 units/mg were analysed by SDS-polyacrylamide gel electrophoresis. Examination of the gel scan (figure 60) however, revealed the possible existence of two molecular species having very similar mobilities and hence similar M_r values. The two components stained with approximately equal intensity using Coomassie Brilliant Blue.

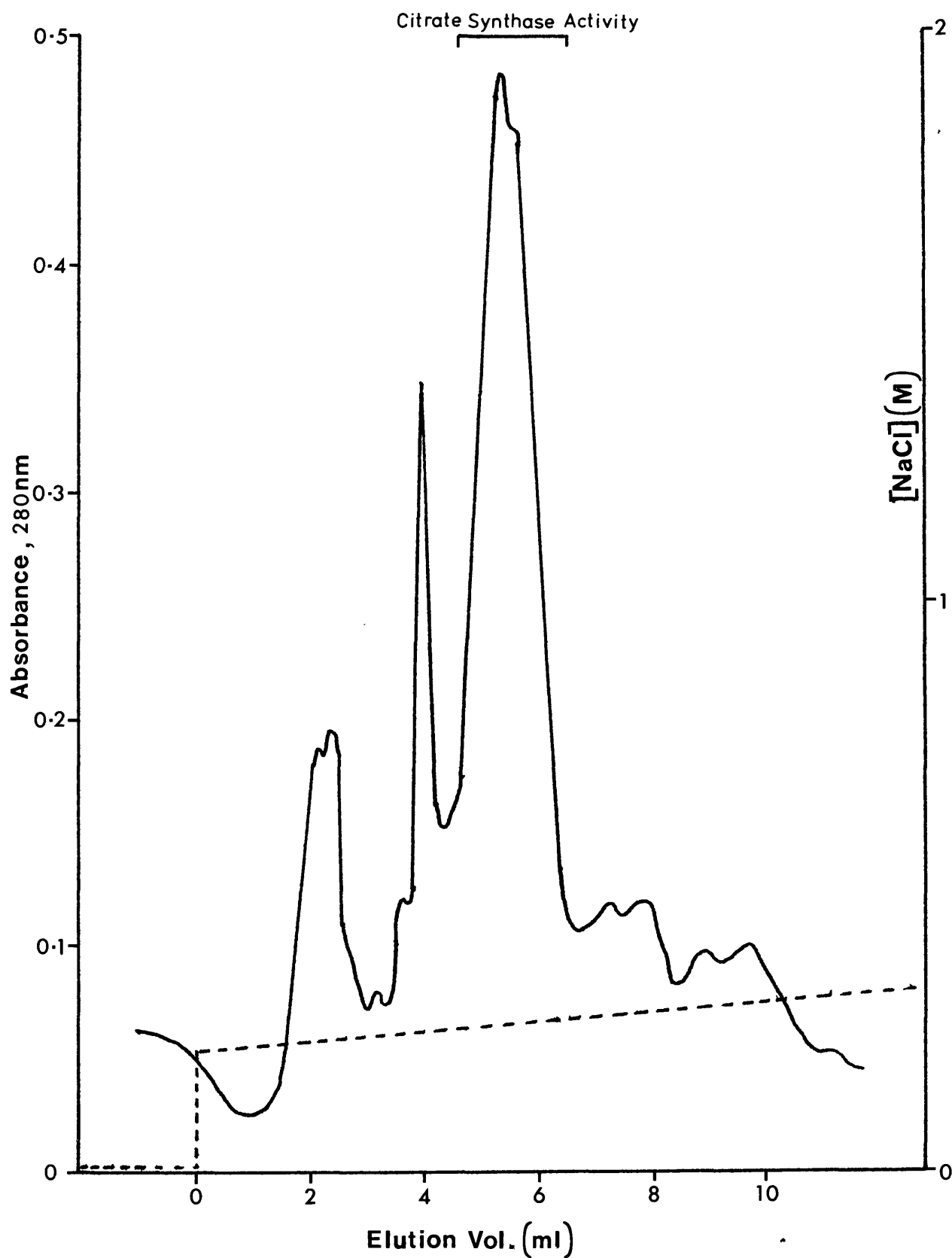


Figure 59. Elution Profile of *E. coli* K114R Citrate Synthase from the Mono Q Anion Exchange Column

Elution was performed using a linear gradient of NaCl (broken line) in 20mM triethanolamine buffer, pH7.3, as described in the text.

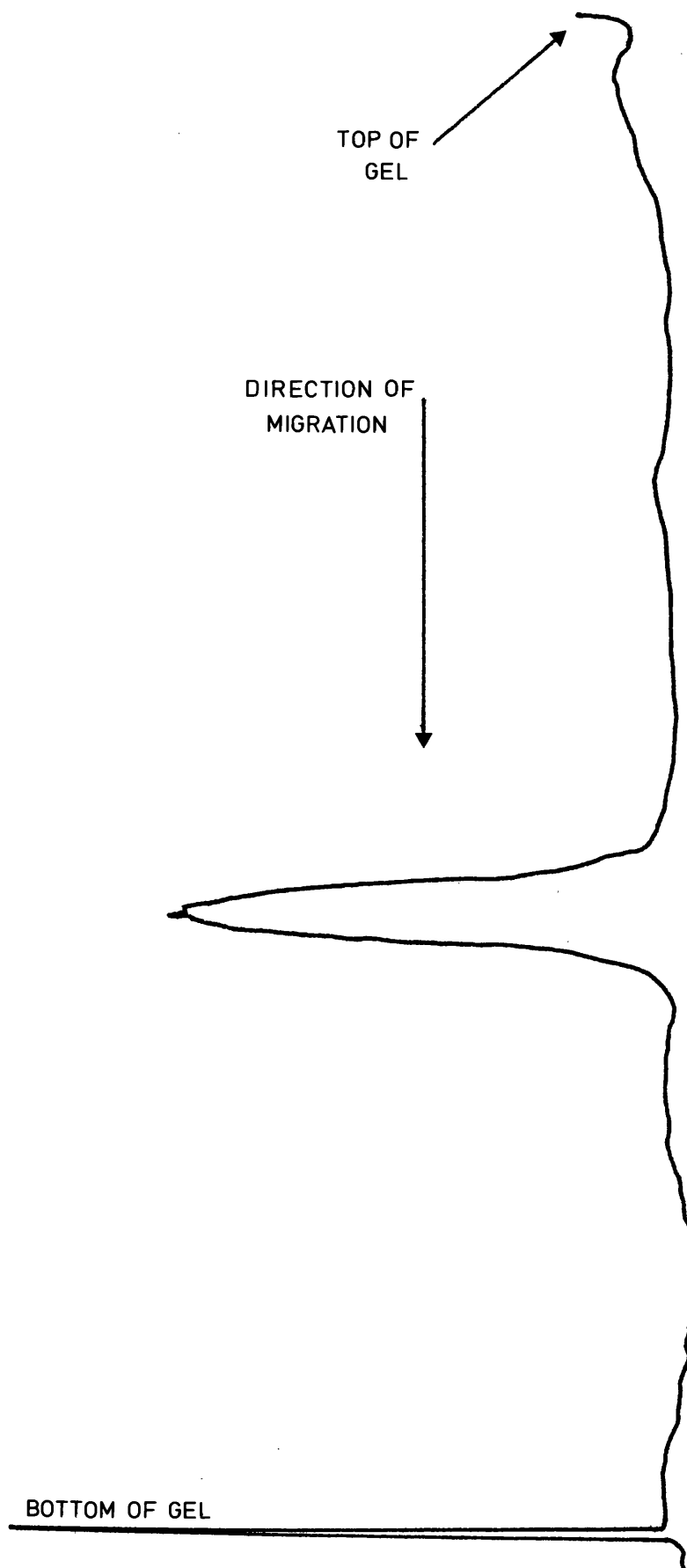


Figure 60 Densitometer Trace of Purified Citrate Synthase from
E. coli K114R after SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis at pH6.7 and staining were performed as described in Methods.

Further purification of the enzyme was not possible since there was insufficient protein remaining after SDS-polyacrylamide gel analysis.

Chromatography using Matrex Gels Red A, Blue B and Orange A was unsuccessful since complete inactivation of E. coli K114R citrate synthase occurred when the enzyme was exposed to these ligands.

Time did not permit a further attempt at purification. The purification procedure is summarised in table 8 and represents a 200-fold increase in specific activity with a 2% yield.

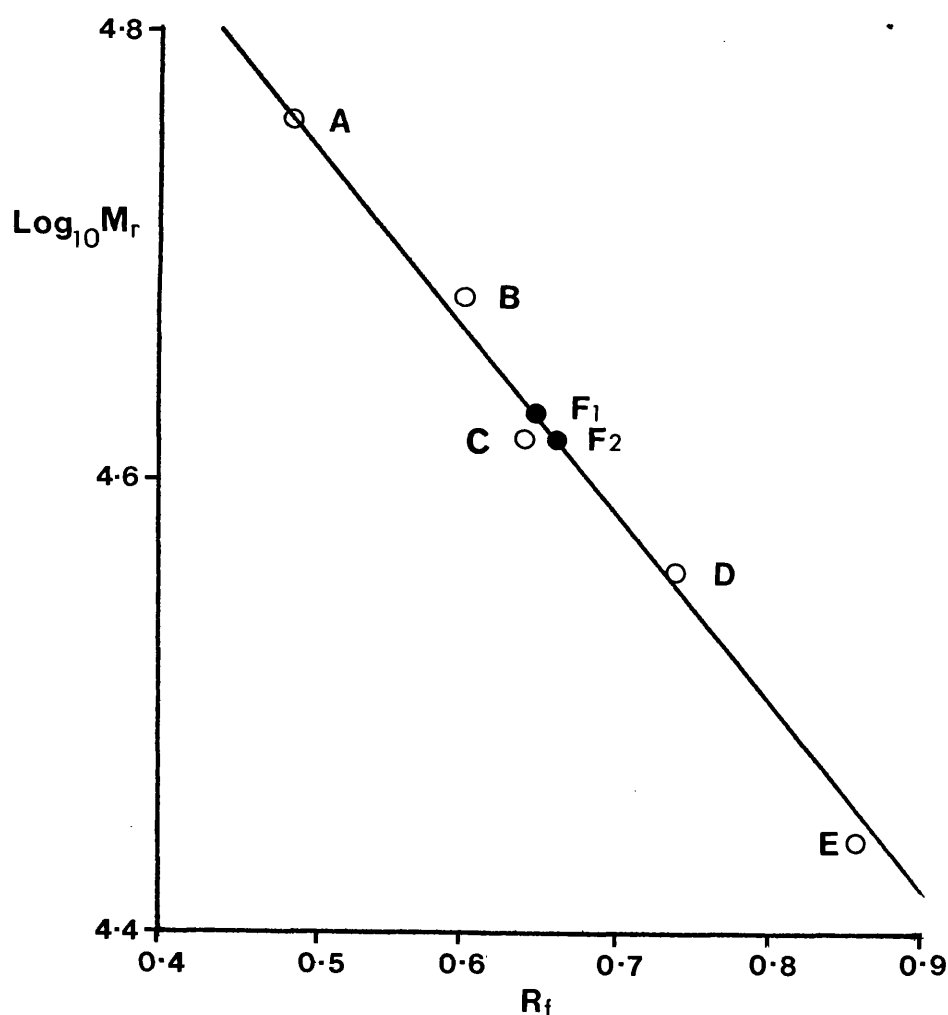
Determination of the Subunit M_r Value

The mobilities of two molecular species of E. coli K114R citrate synthase were compared with those of standard proteins on SDS-polyacrylamide gel electrophoresis. The following proteins were used as standards: pig heart pyruvate kinase, pig heart citrate synthase, pig heart lactate dehydrogenase, rabbit muscle fructose bisphosphate aldolase and yeast triose phosphate isomerase. The results are presented as a plot of $\log_{10} M_r$ versus relative mobility (Rf) in figure 61. The components of E. coli K114R citrate synthase had very similar relative mobilities to aldolase ($M_r=42,000$). The M_r values determined for the two components were 42,600 (± 200) and 42,000 (± 250). Thus if either or both of these molecular species represent the subunit of E. coli K114R citrate synthase the M_r value is 42,000 - 43,000.

Table 8. Purification of Citrate Synthase from *E. coli* K114R

Step	Volume (ml)	Total citrate synthase (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
(1) French press extract	27	58	2014	0.03	-
(2) Supernatant from protamine SO ₄ treatment	29	65	2065	0.03	111
(3) Supernatant from 40% ammonium SO ₄ treatment	30	74	390	0.19	127
(4) Resuspended pellet from 60% ammonium SO ₄ treatment	5	28	110	0.25	47
(5) Pooled, selected fractions from Sephadex G-200	10	5	66	0.08	9
(6) Pooled, selected fractions from Mono Q ion exchange - elution with acetate	5	2.1	2.5	0.84	4
(7) Pooled, selected fractions from Mono Q ion exchange - elution with chloride	0.5	1.1	0.2	5.50	2

Figure 61 SDS-Polyacrylamide Gel Electrophoresis of Citrate Synthase from *E. coli* K114R - Relationship between $\text{Log}_{10} M_r$ and Relative Mobility (R_f).



Electrophoresis was performed at pH6.7 as described in Methods. Proteins used as standards were: (a), rabbit muscle pyruvate kinase (subunit M_r 57,500); (b) pig heart citrate synthase (subunit M_r 49,000); (c), rabbit muscle fructose bisphosphate aldolase (subunit M_r 42,000); (d) pig heart lactate dehydrogenase (subunit M_r 36,000); and (e) yeast triose phosphate isomerase (subunit M_r 28,000). F_1 and F_2 represent the components of *E. coli* K114R citrate synthase. The diameter of the points symbols is greater than the standard errors obtained.

Discussion

The results indicate that the citrate synthase from E. coli K114R has a native M_r of 76,000 and is probably a dimer having subunit M_r values of 42,000 - 43,000.

The subunit M_r value is thus somewhat smaller than that determined for wild-type E. coli citrate synthase (46,000). Not only has the mutation caused the generation of a smaller polypeptide chain, but the subunit-subunit interactions which are necessary to form the wild-type hexameric citrate synthase have also been lost. One explanation of the reduced polypeptide chain length is that the mutation may have caused the generation of a nonsense codon in that part of the glt A gene which corresponds to a section near the C-terminus of the polypeptide chain, thus bringing about premature chain termination in translation. The ease of generation of the mutant enzyme by reversion of citrate synthase deficient strains and the ease of reversion of E. coli K114R to wild-type suggests that a relatively minor change in the glt A gene has occurred.

Of the two molecular components, detected by analysis on SDS-polyacrylamide gel electrophoresis, several possibilities exist. First, only one of the proteins is E. coli K114R citrate synthase in which case the enzyme isolated was approximately 50% pure and therefore is likely to have a specific activity of about 10 units/mg when purified to homogeneity. Second, both components form the mutant enzyme, and either the enzyme has identical subunits and some proteolysis has occurred during the purification procedure, or more unlikely the enzyme is composed of non-identical

subunits. This latter explanation would necessitate the occurrence of a complex mutation mechanism which, as discussed earlier, seems unlikely.

As stated above, the specific activity of pure citrate synthase from E. coli K114R is probably 5-10 units/mg compared with a value of 40-60 units/mg for the wild-type enzyme. This relatively low specific activity is also reflected in the initial cell-free extract and suggests that E. coli K114R may contain a similar weight of citrate synthase per cell as the wild-type organism, but that the mutant enzyme is intrinsically less active by a factor of approximately 3-8 fold.

E. coli K114R grows considerably slower compared with the wild-type organism with a lower yield of cells when grown under the same conditions (2g/l as compared with 3-4g/l for the wild-type), Perhaps the citrate synthase possessed by E. coli K114R is not as well suited to its growth requirements as the wild-type enzyme. Whether this is a reflection of its relatively weak specific activity or its regulatory properties associated with other 'small' citrate synthases rather than those of the E. coli wild-type enzyme remains to be seen.

CHAPTER 5

General Discussion

General Discussion

A summary of the properties of the citrate synthases from wild-type E. coli, mutant E. coli and Bacillus megaterium is shown in table 9. The 'large' citrate synthase from wild-type E. coli was shown to be hexameric and the cross-linking studies indicated that the subunits may be arranged as a trimer of dimers. Observations by Mitchell and Weitzman (1983) on the citrate synthase from the Gram-negative aerobe Acinetobacter calcoaceticus suggested that the enzyme is also hexameric. This may be a general case for 'large' citrate synthases and the purification and characterization of other examples of the enzyme could confirm this.

Of the 'small' enzymes studied in this present work, both appeared to be dimers, although cross-linking studies on the citrate synthase from E. coli K114R are needed to verify that this is so for this enzyme. So far all 'small' citrate synthases appear to be dimeric and this may be a general trend for this category.

The subunit sizes of all the citrate synthases in the present study were found to be similar although small differences were apparent. The M_r values were in the range 42,000 - 49,000 and this seems to be the case for citrate synthases from a wide diversity of sources, whether they be 'large' or 'small' enzymes (Weitzman and Danson, 1976). Since this work was completed the entire sequence of the E. coli glt A gene has been elucidated (Ner et al., 1983). This sequence corresponds to a biosynthetic product of 427 amino acids and a subunit M_r of 48,069 which is in close agreement with the values determined by SDS-polyacrylamide gel electrophoresis.

Table 9. Comparison of the Properties of Citrate Synthase from
E. coli (Wild-Type and Mutant) and *B. megaterium*

	<u><i>E. coli</i></u> (wild-type)	<u><i>E. coli</i></u> (mutant)	<u><i>B. megaterium</i></u>
Enzyme M_r	230,000 - 270,000	76,000	84,000
Subunit M_r	47,000	42,000 - 43,000	39,000 - 43,000
Number of subunits	6	2 ?	2
Substrates dependences	Sigmoidal	Hyperbolic	Hyperbolic
Oxaloacetate K_m or $S_{0.5}$ (μM)	78 ^a	12 ^a	9
Acetyl-CoA K_m or $S_{0.5}$ (μM)	359 ^b	4 ^b	88
ATP K_i (μM)	N.D.	205	1100
Inhibition by 0.1mM NADH (%)	90 ^c	0	0
Inhibition by 1mM 2-oxo- glutarate (%)	79	0	0

All K_m values quoted are calculated at infinite concentration of the second substrate except (a), at 150 μM acetyl-CoA; and (b), at 200 μM oxaloacetate; (c) from Danson et al. (1979 b).

and gel filtration in guanidinium chloride.

The kinetic properties displayed by the citrate synthase from B. megaterium and E. coli K114R are in keeping with those shown by other dimeric citrate synthases, namely the hyperbolic substrate dependences and the isosteric, competitive inhibition by ATP with respect to acetyl-CoA.

Neither enzyme was sensitive to 2-oxoglutarate or NADH. The observations of the properties of the dimeric mutant citrate synthase from E. coli K114R compared to those of the hexameric wild-type enzyme suggest that allosteric inhibition by NADH of wild-type citrate synthase is brought about by the subunit interactions of the hexameric enzyme. When conversion of hexamer to dimer occurs, these subunit-subunit interactions are lost and an isosteric binding site for ATP is gained. The change in configuration which provides a binding site for ATP may also be responsible for the lower K_m value for acetyl-CoA. This finding is in keeping with the unifying hypothesis for nucleotide inhibition of citrate synthase suggested by Weitzman and Danson (1976). In general those citrate synthases isosterically inhibited by ATP have low K_m values for acetyl-CoA whereas those insensitive to ATP have relatively high K_m values.

The comparison of amino acid sequences of citrate synthase from pig heart and E. coli (wild-type) shows a large degree of homology, particularly with regard to the residues at the active site (Ner et al., 1983). However, it was observed that the region where the polypeptide chain displayed only fortuitous homology was at the amino terminus (residues 1 - 100). Ner et al. (1983)

concluded that these structural differences in the amino terminal region may be responsible for the diverse properties exhibited by the hexameric and dimeric citrate synthases, particularly with respect to the binding of ATP. This view is not supported by the evidence from comparisons of the subunit M_r values of citrate synthase from the wild-type and mutant strains of E. coli. The subunit M_r of the mutant E. coli K114R citrate synthase appeared to be some 5,000 less than that of the wild-type enzyme. As discussed earlier this may represent the loss of some 50 - 60 amino acid residues from the carboxy terminal region rather than the amino terminus. Sequence comparisons of both wild-type and mutant citrate synthases are necessary however before more definite conclusions can be drawn.

Histidine residues have been shown to have important roles in the catalytic activity of citrate synthase from E. coli (wild-type) and pig heart (Weitzman and Danson, 1973; Mählen, 1975). Observations in the current work revealed that B. megaterium citrate synthase also has a histidine residue crucial for activity and that it is probably very near or at the active site. Recent X-ray crystallographic studies on the citrate synthase from pig heart enabled Remington et al. (1981) to build a picture of the active site. Significantly, two histidine residues were found to play important roles in the catalytic cycle. One residue donates a proton to the keto carbonyl group of oxaloacetate and the other removes a proton from the incoming methyl group of acetyl-CoA. This allows the methyl group to attack the si face of oxaloacetate forming the intermediate citryl-CoA, which is then subsequently

hydrolysed to produce citrate and CoA.

Pig heart citrate synthase has been shown to exhibit two different forms in the catalytic cycle; a hydrolase configuration representing the free enzyme, and a ligase form (Bayer et al., 1981). Oxaloacetate binds to the free enzyme first (Johansson and Petersson, 1974) converting the configuration from a hydrolase to ligase (Bayer et al., 1981). Reconversion to a hydrolase is achieved upon the formation of citryl-CoA after the binding of the second substrate, acetyl-CoA. This kinetic pattern is not shown by the citrate synthases from other eukaryotic organisms. In contrast to the pig heart enzyme, citrate synthase from the fungus Aspergillus niger also displays an ordered sequential mechanism but with acetyl-CoA binding first to the enzyme, followed by oxaloacetate (Kubicek and Röhr, 1980). This is also true for the citrate synthase from the slime mould Dictyostelium discoideum (Porter and Wright, 1977). These differences in the order of addition of substrate may reflect variations in the active sites of citrate synthases and more detailed kinetic analysis of the enzymes from both prokaryotes and eukaryotes may give additional information regarding evolutionary relationships. Indeed, there is evidence to suggest that the endosymbiotic eubacterial origin of fungal mitochondria was independent of the bacterial origin of animal mitochondria. This is based on the comparison of the sequences of the small ribosomal subunit RNA (s-r RNA) genes from E. coli, fungal and mammalian mitochondria and nuclei (Küntzel and Köchel, 1981).

A comparison of the amino acid compositions of citrate synthase from pig heart (Bloxham et al., 1981), wild-type E.coli (present study and Ner et al., 1983) and B. megaterium (present study) is shown in table 10. The "composition divergence" (D) was determined

Table 10. Comparison of the Amino Acid Composition of Citrate

Synthase from Pig Heart, *E. coli* (Wild-Type) and

B. megaterium

Amino Acid	Pig Heart	<u><i>E. coli</i></u>		<u><i>B. megaterium</i></u>
	(Bloxham <u>et al.</u> , 1981).	(Present study)	(Ner <u>et al.</u> , 1983)	(Present study)
Alanine	33	36	37	40
Arginine	19	23	24	19
Asparagine] 40] 42	17]] 35
Aspartate			27]	
Cysteine	4	7	7	4
Glutamine] 41] 36	8]] 53
Glutamate			26]	
Glycine	33	30	28	38
Histidine	14	15	15	16
Isoleucine	19	25	28	17
Leucine	53	34	35	35
Lysine	25	22	23	19
Methionine	15	14	19	8
Phenylalanine	12	20	23	10
Proline	22	18	19	16
Serine	29	26	25	35
Threonine	23	27	28	23
Tryptophan	9	2	3	1
Tyrosine	19	14	16	11
Valine	28	20	19	20
Total	<u>437</u>	<u>411</u>	<u>427</u>	<u>400</u>
Subunit total M _r	48,969	45,130	48,069	41,984

from the following equation (Harris and Teller, 1973):

$$D = \sqrt{\sum (X_{iA} - X_{iB})^2}$$

where X_{iA} and X_{iB} are the mole fractions of the same amino acid residue in proteins A and B respectively. A value of D less than 7.0×10^{-2} indicates a non-fortuitous similarity of composition (Harris and Teller, 1973). Curiously although the citrate synthases from pig heart and E. coli differ widely in structural and kinetic properties they displayed the closest similarity ($D = 6.0 \times 10^{-2}$). B. megaterium citrate synthase and that from pig heart exhibited a possible fortuitous similarity in composition ($D = 7.2 \times 10^{-2}$). However until the amino acid sequence of B. megaterium citrate synthase is determined such comparisons are open to doubt.

In conclusion the properties of the citrate synthases investigated in the present study indicate that the 'large' enzyme from E. coli (wild-type) is hexameric. The 'small' enzyme from a Gram-positive bacterium has been purified to homogeneity and shown to be dimeric, the first occasion this has been demonstrated with citrate synthase from a Gram-positive organism. Protein modification and kinetic studies have also shed some light on the possible role of histidine residues in the catalytic activity of the enzyme and variation in the kinetic properties of B. megaterium citrate synthase with pH has been shown. A citrate synthase resembling those from Gram-positive bacterial and eukaryotic sources has been partially purified from a mutant strain of E. coli. The possible genetic changes and alterations in protein structure have been discussed in the light of the finding that the enzyme is dimeric and sensitive to

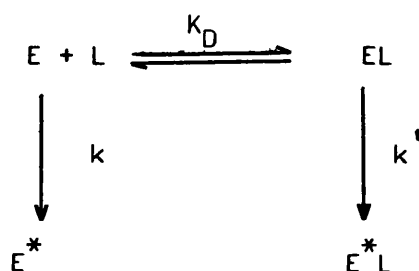
ATP in contrast to the hexameric, NADH sensitive wild-type E. coli citrate synthase. Site directed mutagenesis of the E. coli qlt A gene may soon be possible and should provide a valuable tool in elucidating those amino acid residues which play crucial roles in the maintenance of a hexameric NADH-sensitive enzyme. By selective mutations interconversions between the two forms may be achieved.

Both 'small' and 'large' types of citrate synthase have been detected in the same organism in several strains of Pseudomonas (a Gram-negative aerobe) (Solomon and Weitzman, 1983; C.G. Mitchell, personal communication) although the genetic basis for this phenomenon has not yet been investigated.

Studies on other citric acid cycle enzymes from diverse sources are continuing in Professor Weitzman's and Dr. Danson's laboratories in order to give further insights into evolutionary trends and relationships. Some of the organisms under investigation are the Archaeobacteria which are genealogically neither prokaryotes nor eukaryotes (Woese, 1981). Their metabolism and life-styles are particularly suited to the conditions which prevailed on earth when life first began and isolation and investigation of the citric acid cycle enzymes from these organisms may shed further light on the pattern of evolution. Advances in recombinant DNA techniques are also proving a useful complementary tool to the study of enzyme protein per se and may accelerate advances in the elucidation of structure-function relationships of citric acid cycle enzymes in the future.

Appendix

Protection of an enzyme by ligands against chemical modification can be described by the following relationship:



where E is unmodified, active enzyme; E^* is the modified enzyme; L is the ligand; K_D is the dissociation constant of the enzyme-ligand complex; k is the rate constant for the modification of free enzyme; and k' is the rate constant for the modification of ligand-bound enzyme.

$$[E_t] = [E] + [EL] + [E^*] + [E^*L] \quad (1)$$

where $[E_t]$ is the total enzyme concentration.

$$[EL] = [E][L] / K_D$$

Substituting for $[EL]$ into equation 1:

$$[E_t] = [E] (1 + [L] / K_D) + [E^*] + [E^*L]$$

and rearranging:

$$[E] = \frac{[E_t] - [E^*] - [E^*L]}{(1 + [L] / K_D)} \quad (2)$$

$$\text{Rate of inactivation} = k' [EL] + k [E]$$

substituting for $[EL]$:

$$\text{Rate of inactivation} = k' [E] [L] / K_D + k [E]$$

substituting for $[E]$ from equation 2:

$$\text{Rate of inactivation} = \frac{k + k' [L] / K_D ([E_t] - [E^*] - [E^*L])}{1 + [L] / K_D}$$

Collecting together constants:

$$k_{\text{obs}} = \frac{k + k' [L] / K_D}{1 + [L] / K_D}$$

and rearranging:

$$\frac{1}{k_{\text{obs}} - k} = \frac{K_D}{k' - k} \left[\frac{1}{[L]} \right] + \frac{1}{k' - k}$$

Thus plotting $\frac{1}{k_{\text{obs}} - k}$ versus $\frac{1}{[L]}$ will give an intercept

of $\frac{1}{k' - k}$ and a gradient of $\frac{K_D}{k' - k}$

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